The Purification and Characterisation of a Cysteine Specific Mono-ADP-ribosyltransferase from Bovine Erythrocytes.

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PREFACE

The work presented in this thesis was carried out between 1st October 1990 and 30th September 1993. The work was performed under the supervision of Dr Simon van Heyningen at the department of Biochemistry, University of Edinburgh Medical School, in Edinburgh. All material presented in this thesis is the sole work of the author, as is the composition.

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ABSTRACT

Mono-ADP-ribosyltransferases, which have activities similar to bacterial exotoxin ADP-ribosyltransferases, have been identified in a variety of tissues. Few have been purified to homogeneity and of these arginine specific cholera toxin-like ADP-ribosyltransferases are most common. In this thesis the purification and characterisation of a cysteine specific pertussis toxin-like mono-ADP-ribosyltransferase is reported. This protein was purified on the assumption that, like pertussis toxin, the enzyme would be able to use free cysteine as an acceptor for ADP-ribose in the absence of its protein substrate. A cysteine specific ADP-ribosyltransferase activity was found in bovine erythrocytes. A three step purification procedure was developed involving (1) precipitation with 40 % ammonium sulphate, (2) binding to a cysteine Sepharose affinity column, and (3) binding to a NAD^+ affinity column. Polyacrylamide gel electrophoresis showed a single band at 45,000. The enzyme had been purified 47000 fold and had a specific activity of 1900 nmol nicotinamide released min^{-1} mg^{-1}. A study of the kinetic properties of this enzyme showed that apparent substrate kinetics for cysteine were observed (K_m of 4.4 mM) and a putative ADP-ribosyl-cysteine product was identified by HPLC. The ability of this enzyme to ADP-ribosylate protein was investigated using re-sealed inverted bovine erythrocyte ghosts. Incubation of the purified enzyme with erythrocyte ghosts and [adenylate-^{32}P]NAD^+ led to the enhanced labelling of a 55 kDa protein identified by autoradiography of separated proteins on SDS-polyacrylamide gels. Pertussis toxin also caused enhanced labelling of this 55 kDa protein as well as labelling of a 42 kDa protein, $G_i\alpha$. The 55 kDa protein was not recognised by antibodies raised against G_i or G_s and has not been identified. However evidence that mono-ADP-ribosylation of a cysteine residue on this 55 kDa protein had been catalysed was supported in three ways. (1) the labelling was blocked by pre-treatment of the erythrocyte ghosts with N-ethylmaleimide (NEM), a sulphydryl alkylating agent. (2) the labelling was insensitive to hydroxylamine, but was released by mercuric ion, and (3) the released radiolabelled product was identified as ADP-ribose on HPLC.

TABLE OF CONTENTS

Chapter five **ADP-ribosyltransferase Activity**

Chapter six **Conclusion and Future Work**

ABBREVIATIONS

DTNB	5,5'-dithiobis(2)nitrobenzoic acid
ECL	enhanced chemiluminescence
NAD'ase	nicotinamide adenine dinucleotide glycohydrolase activity
OPA	*ortho*-phthaldialdehyde
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PMSF	phenyl methyl sulphonyl sulphate
PVDF	poly vinylidene di-fluoride
TBS	Tris buffered saline
TCA	trichloroacetic acid

INTRODUCTION

NAD^+ dependent ADP-ribosylation of cellular proteins is an important post-translational modification by which protein structure and function may be altered. Two classes of ADP-ribosylation reaction occur, poly-ADP-ribosylation and mono-ADP-ribosylation. These reactions differ not only in the chain length of the ADP-ribose units, but also with respect to the chemical nature of the glycosidic linkage, the enzymes involved and the site of reaction.

Poly-ADP-ribosylation is primarily associated with the eucaryotic nucleus and has an important role in regulation of nuclear events, eg DNA repair, chromatin organisation, cellular differentiation and proliferation[1]. The work presented in this thesis however is concerned with mono-ADP-ribosylation. Mono-ADP-ribosyltransferase activity was first discovered during the study of the action of some bacterial protein exotoxins and has been known for some time. These systems have been well characterised. Study of the action of these bacterial toxins on eukaryotic cells indicated that endogenous mono-ADP-ribosylation existed. Indeed mono-ADP-ribosylated protein predominated by far over poly-ADP-ribosylation in mammalian cells outside of the nucleus. It is only recently that endogenous mono-ADP-ribosyltransferases have been purified from eukaryotic cells, however the physiological role of these reactions is poorly understood.

Mono-ADPribosyltransferases (E.C. 2.4.2.30 and E.C. 2.4.2.31) catalyse the transfer of a single ADP-ribose unit from NAD^+ to an acceptor site on a target protein with the concomitant release of nicotinamide and a proton:

$$NAD^+ + \text{protein} \rightleftharpoons \text{ADP-ribosyl-protein} + \text{nicotinamide} + H^+$$

The bacterial exotoxins studied are specific for a particular amino acid of a particular target protein. The endogenous enzymes purified to date also appear to be site and target specific. ADP-ribosylation of arginine residues have been most commonly reported, but modification of lysine, asparagine, modified histidine (diphthamide) and cysteine residues are also observed. Many of the bacterial exotoxins studied in detail exert their action on a single family of GTP binding

proteins, the G proteins. It is remarkable that entirely different organisms should each secrete an enzyme activity, for which no endogenous role has been attributed within the bacterial metabolism, which can modify a related family of proteins in eucaryotic cells by apparently similar molecular mechanisms. Structure function studies of these enzymes shows very little homology at the amino-acid level although essential catalytically active residues are conserved. Crystal structures of some of the bacterial toxins have been resolved, and it is speculated that a NAD^+ binding groove is common to all ADP-ribosylating toxins. Endogenous mammalian enzymes which apparently catalyse the same reaction have been identified, but the extent of knowledge of these enzymes is very limited. Much of the data concerning endogenous mammalian ADP-ribosylation comes from identification of ADP-ribosylated proteins after various stimuli, eg hormones, nitric oxide or nutritional stress. ADP-ribosylation is observed in a variety of cells and the target proteins are found in a variety of cellular compartments, eg nucleus, mitochondria, cytosol, plasma-membrane or cytoskeleton. ADP-ribosylation has been associated with a variety of biological processes involving receptor-mediated signal transduction Ca^{2+} signalling and growth and differentiation. How ADP-ribosylation is switched on or off is not clear. Reversible ADP-ribosylation as a mechanism for controlling metabolism has been demonstrated in a prokaryotic system. Enzymes which specifically cleave ADP-ribosyl-protein have been purified from mammalian cells, which allows for a reversible control mechanism.

1.1 Bacterial Protein Exotoxins

Mono-ADP-ribosylation was first identified on discovery of the mechanism of action of diphtheria toxin twenty five years ago[2]. Diphtheria toxin is secreted by *Corynebacterium diphtheriae* and is the causative agent of the disease. The target of the toxin is elongation factor 2 (EF-2) and its ADP-ribosylation results in inhibition of protein synthesis. The site of ADP-ribosylation is a modified histidine residue called diphthamide, which was identified by van Ness[3]. Mono-ADP-ribosylation was also found to be of importance in the pathogenesis of cholera and whooping cough. Cholera toxin is secreted by *Vibrio cholerae* and its intra-cellular target is the

stimulatory component of adenylate cyclase, G_s. ADP-ribosylation of G_s results in the permanent activation of adenylate cyclase such that cyclic AMP levels rise within the cell and signal transduction is disrupted. Pertussis toxin, secreted by *Bordetella pertussis*, has a similar effect on the cell. The cyclic AMP dependent signalling transduction pathway is disrupted by the permanent activation of adenylate cyclase. The target for ADP-ribosylation is the inhibitory component of adenylate cyclase G_i. (This process is discussed in more detail in section 1.2.5.)

The examples sited above have been well characterised, mainly because of their importance in the pathogenesis of the diseases caused by the organisms. Their mechanism of action, their primary amino acid sequence and even their three dimensional structures are known. In addition cholera toxin and pertussis toxin have provided invaluable tools for studying the interaction of hetero-trimeric G proteins and receptor mediated signalling transduction pathways. Secretion of ADP-ribosylating proteins is not limited to these pathogenic organisms. Many bacteria have been shown to secrete ADP-ribosyltransferases, and these are listed in table 1.1 below. Some have a similar action to those described above, eg pseudomonas exotoxin A and *E. coli* heat labile toxin and others, eg botulinum C2 and C3, modify novel target proteins. *Clostridium botulinum* secretes several neurotoxins, termed A,B,C1,D,E,F and G which are all antigenically different. The ADP-ribosylating toxins C2 and C3 secreted by certain strains are distinct from the neurotoxins in that they do not block secretion, instead they induce cellular morphological changes[4]. The substrate for C2 is monomeric actin. ADP-ribosylation of actin inhibits the ATP hydrolysing activity (ATPase) of actin which is essential for polymeristion to actin filaments. The cellular target for C3 is the small GTP-binding protein *rho* p21. C3 catalyses the transfer of ADP-ribose from NAD^+ to form a novel ADP-ribosyl linkage via an asparagine residue of *rho*[5]. For the purposes of this thesis the mechanism of action of pertussis toxin will be discussed in more detail, and structural and functional similarities with other toxins will be identified.

Table 1.1 Bacterial ADP-ribosylating Toxins

BACTERIAL TOXIN	TARGET PROTEIN	ACCEPTOR AMINO ACID	EFFECT ON EUKARYOTIC CELLS
Diphtheria toxin	Elongation factor 2	diphthamide - 715	Inhibition of protein synthesis
Pseudomonas exotoxin A	Elongation factor 2	diphthamide - 715	Inhibition of protein synthesis
Pertussis toxin	α subunit G_i	cysteine - 352	Block receptor mediated signal transduction
Cholera toxin	α subunit G_s	arginine - 201	Block receptor mediated signal transduction
E. coli heat labile toxin	α subunit G_s	arginine - 201	Block receptor mediated signal transduction
Botulinum C3 exoenzyme	*Rho*	asparagine - 41	Micro-injection causes changes in cellular morphology
Staphylococcus aureus, EDIN	*Rho* and *Rac*	unknown	Hyperplasia of the epidermis
Pseudomonas exoenzyme S	*Ras*, vimentin	arginine - ?	unknown
Botulinum C2 toxin	non-muscle actin	arginine - 177	Inhibits actin polymerisation
Clostridium perfringens iota toxin	Monomeric skeletal and non-muscle actin	arginine - 177	Inhibits actin polymerisation
Clostridium spiroforme toxin	Non-muscle actin	unknown	inhibits actin polymerisation
Clostridium difficile transferase	unknown	unknown	Inhibits actin polymerisation

1.2 Pertussis Toxin

Pertussis toxin is one of many proteins secreted by *Bordetella pertussis*, the pathogenic bacterium causing whooping cough. It was first purified in 1978[6] and identified as a 117 kDa protein by gel filtration chromatography. It was referred to as islet activating protein (IAP) after its ability to enhance insulin secretion in pancreatic islets of Langerhan cells[7]. Later it was termed pertussis toxin, reflecting its importance as the major causative agent of whooping cough. ADP-ribosyltransferase activity was first demonstrated as the mechanism of action of the toxin in the early eighties[8]. ADP-ribosylation of a 41 kDa membrane protein was observed in pancreatic islet cells and it was subsequently shown that a cysteine residue was the site of modification[9]. This is the only bacterial toxin known to modify a cysteine residue. In 1986 the cloning of the pertussis toxin operon was achieved independently by two groups[10,11] and little identity with other ADP-ribosylating toxins was noted. The essential regions of the toxin required for activity were mapped and this work paved the way for the development of acellular recombinant DNA derived vaccines to replace the original whole cell preparations[12].

1.2.1 Subunit Organisation

Pertussis toxin is a hexaemeric protein and follows the classical AB type model of bacterial toxins[13]. It has an A protomer which is enzymatically active and a B oligomer which is concerned with attachment and internalisation of the toxin into the host cell. Pertussis toxin has the most complex subunit organisation of these AB type bacterial toxins, in that it is made up of five different subunits. S1 (28,000) catalyses ADP-ribosyltransfer of ADP-ribose moiety from NAD^+ to a target acceptor and is discussed in more detail in section 1.2.3. The B oligomer is made up of four different subunits S2, S3, S4 and S5 (23,000, 22,000, 11,700 and 9,300) in the molar ratio 1:1:2:1. Cholera toxin and *E. coli* heat labile toxin are also hexaemeric proteins, however the B oligomer is made up of one repeating subunit.

The three dimensional crystal structure of *E. coli* heat labile enterotoxin showed that the B oligomer forms a ring doughnut-like structure with the A protomer

attached via an hairpin like structure, made up of the C-terminal residues 222-228, which passes through the centre of the highly charged pore.[14]. *E. coli* heat labile toxin is 80 % identical to cholera toxin at the amino acid level. Preliminary three dimensional structures of cholera toxin appear to show similar subunit arrangement[15]. The complex subunit composition of pertussis toxin made the assignment of the three dimensional structure very challenging. Stein and Reed (Edmonton, Canada) have succeeded[16]. On a structural level pertussis toxin looks like the cholera related toxins, despite the lack of homology between their primary structures. The B oligomer forms a ring structure with an internal diameter 11-15 Å, too small for the A protomer to pass through. The small C-terminal tail inserts into the centre of the ring, but unlike the A protomer of cholera toxin plays little part in holding the B oligomer and A protomer together. The S1 subunit is held tightly associated to the B oligomer by favourable interactions between S1 and S2, S3, S4 and S5.

Diphtheria and related toxins on the other hand appear to be quite different. Diphtheria toxin is secreted as a single poly-peptide which is "nicked" very easily by host proteolytic enzymes to release an active protomer. The idea of separate domains within the single poly-peptide is clearly visualised from the three dimensional crystal structure[17]. Three domains are identified; domain I, receptor binding, domain II, translocation, and domain III, enzymatic activity.

1.2.2 Binding Entry and Activation of Pertussis Toxin

The nature of the receptor site and mechanism of internalisation of the toxin into the cell have not been well characterised. The current view on binding and entry of pertussis toxin has been proposed by Kaslow and Burns[18] and is shown in Figure 1.1. Galactose glycoconjugate and sialic acid glycoconjugate recognition sites have been identified in S2 and S3 subunits of the B oligomer which may serve to anchor the toxin at the cell surface[19]. Two possible mechanisms for internalisation of bacterial toxins have been proposed, endocytosis and direct penetration. An example of a toxin which is enters by endocytosis is diphtheria toxin[20]. Cholera toxin is thought to penetrate the membrane directly[21] and it is thought that pertussis toxin proceeds in a similar fashion. The molecular basis for this mechanism is not understood.

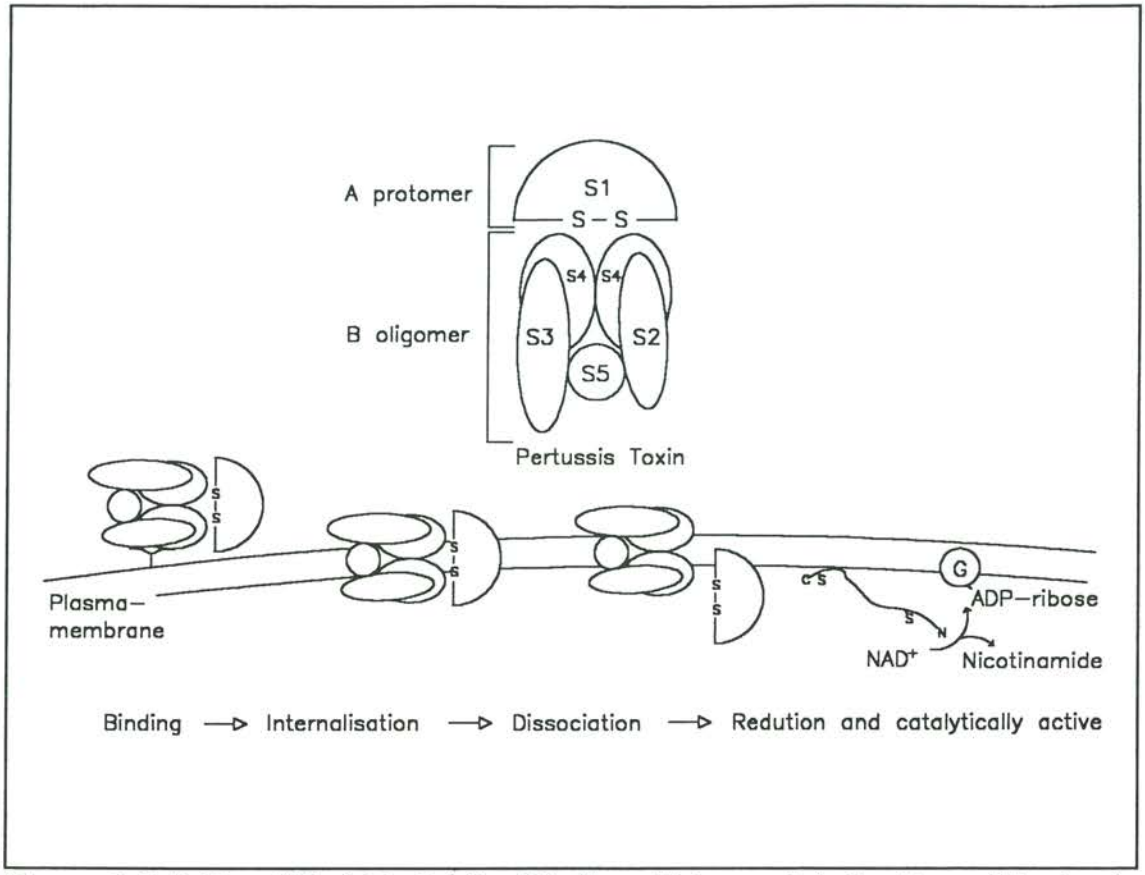


Figure 1.1 A Simplified Model for Binding, Entry and Activation of Pertussis Toxin.

Before ADP-ribosyltransferase activity is observed the toxin must undergo an activation process which involves the release of S1 from the B oligomer and reduction of an intra-molecular disulphide. Kaslow *et al.*[22] proposed a model for activation in which ATP and lipophilic substances bind to the whole toxin and promote the release of S1 which is then reduced and can exert its toxic effect on its protein target. Cellular concentrations of ATP and glutathione would be sufficient to promote this process such that host enzymatic machinery would not be required for the activation process. The C-terminal tail of S1 is highly hydrophobic and it is thought it might anchor the S1 to the membrane rather than allowing its release into the cytoplasm[23].

The role of the B oligomer is not simply a carrier for targeting the ADP-ribosyltransferase activity of S1. The B oligomer also has some intrinsic activity of its own, independent of ADP-ribosylation, which are manifested in the disease state eg lymphocytosis promotion, histamine sensitisation and haemagglutination[24]. *In vitro* experiments with Jurkat T lymphocyte cell lines have shown increases in inositol

phosphate and intra-cellular calcium concentrations on treatment with the B oligomer alone[25].

1.2.3 The S1 Subunit

The S1 subunit catalyses the transfer of ADP-ribose moiety from NAD^+ to a C-terminal cysteine residue of $G_i\alpha$[9] with the concomitant release of nicotinamide and a proton. In the absence of the target protein the toxin may use water as an acceptor for ADP-ribose. This abortive reaction is termed the NAD glycohydrolase activity and is common to all the ADP-ribosylating toxins. This reaction is a useful tool for studying the mechanism of the enzyme and is discussed in more detail in chapter four.

The amino-acid sequence of the S1 subunit has been compared to the active subunits of the other known ADP-ribosylating toxins[10,11]. Two regions, eight amino acids each, showing significant homology can be identified between pertussis toxin, cholera and *E. coli* heat labile toxin.

REGION 1

Pertussis toxin	S1	8	Tyr	Arg	Tyr	Asp	Ser	Arg	Pro	Pro	15
Cholera toxin	A	6	Tyr	Arg	Ala	Asp	Ser	Arg	Pro	Pro	13
E. coli LT	A	6	Tyr	Arg	Ala	Asp	Ser	Arg	Pro	Pro	13

REGION 2

Pertussis Toxin	S1	51	Val	Ser	Thr	Ser	Ser	Arg	Ser	Arg	58
Cholera toxin	A	60	Val	Ser	Thr	Ser	Leu	Ser	Leu	Arg	67
E. coli LT	A	60	Val	Ser	Thr	Ser	Leu	Ser	Leu	Arg	67

These similarities at the amino acid level are not mirrored in the DNA sequences as pertussis toxin uses different triplet codons to that of cholera toxin and *E. coli* LT. No other sequence similarities were found between the toxins.

A putative "Rossman fold"[26], the phosphate binding site of nucleotide binding proteins, was identified in the C-terminal region of S1 from the sequence motif G-X-G-X-X-A[27]. However this region was found not to be essential for enzymatic activity following the construction of deletion mutants. The C-terminal portion of S1, amino

acids 181-234[27], are not essential for enzymatic activity however ADP-ribosylation is more efficient with whole S1[28]. This apparent differentiation of S1 subunit into two domains is similar to the differentiation of cholera toxin A protomer into A1 (N-terminal, catalytically active) and A2 (C-terminal). Photoaffinity labelling experiments with NAD^+ identified an interaction with glu-129[29]. Comparable experiments with cholera and diphtheria toxins also showed modification of a glutamate residue. Substitution of glu-129 for glycine or aspartic acid residues by site directed mutagenesis inactivated the toxin suggesting a catalytic role for this residue[30]. Other important residues have been identified in the N terminal portion of the subunit which probably have a role in binding of NAD^+: deletion or conservative substitution of these aminoacids causes an increase in the affinity of binding NAD^+ but has no effect on the maximal rate of catalysis. These include amino acids tyr-8 to pro-15[31] (region 1), trp-26[32] and cys-41[33].

These studies suggested that there may be a common NAD^+ domain among the ADP-ribosylating toxins. The resolution of the crystal structures of Pseudomonas exotoxin A and E. coli LT support this idea[34]. Structural superposition of the enzymatic domains showed significant structural similarity. This is remarkable given the lack of sequence identity at the amino acid level. 44 residues, only three of which were identical from the central region of the molecule were superimposed upon one another. These residues corresponded to tyr-6, ala-69 and glu-112 in *E. coli* heat labile toxin.

1.2.4 Protein Substrates of Pertussis Toxin

The main protein substrate for ADP-ribosylation by pertussis toxin is G_i, a member of the heterotrimeric guanine nucleotide-binding protein family (G proteins). G proteins act as switches that regulate infomation processing circuits connecting cell surface receptors to a variety of effectors. The G proteins are present in all eucaryotic cells, and they control metabolic, humoral, neural and developmental functions. More than a hundred different receptors and many different effectors have been described. The G proteins are made up of three different subunits α, ß and γ (39 - 52 kDa, 35 kDa and 8 kDa respectively) that are derived from a large gene family. This family contains at least sixteen different genes encoding the α subunit,

four that encode the ß subunit and multiple genes encoding the γ subunit. Pertussis toxin sensitive Gα subunits are characterised by a conserved C-terminal cysteine residue and are listed in table 1.2.

Table 1.2 Pertussis Toxin Sensitive and Insentive Gα Subunits

G PROTEIN	C-TERMINAL SEQUENCE	SUBSTRATE FOR PERTUSSIS TOXIN	REFERENCE
$G_i\alpha$	LKDCGLF	+	35
$G_{i2}\alpha$	LKDCGLF	+	35
$G_{i3}\alpha$	LKECGLY	+	35
$G_{t1,2}\alpha$	LKDCGLF	+	35
$G_{o1,2}\alpha$	LRGCGLY	+	36
Gsα	LRQYELL	-	37
Gαq, Gα11	LREFNLV	-	37

G_i is involved in the regulation of adenylate cyclase, which generates the important second messenger cyclic AMP, and is normally under the control of inhibitory hormones. Adenylate cyclase is also under the control of a stimulatory regulatory GTP-binding protein, G_s which is the substrate for cholera toxin. The phospholipase C enzyme is another protein under the control of GTP binding proteins[38] which may be uncoupled from its receptor by the action of pertussis toxin. Phospholipase C is important for the production of two second messengers - inositol triphosphate and diacylglycerol, within the cell. These G proteins are known as G_p and at least two forms exist, one of which is a substrate for pertussis toxin catalysed ADP-ribosylation[39]. Transducin is closely related to G_i and is found in the outer rod segments of the eye. Its role is to mediate signal transduction from the light receptor rhodopsin to cGMP phosphodiesterase which raises the concentration of cGMP in the

retinal rod cell resulting in the hyperpolarisation of the rod membranes and a change in neurotransmitter release of the output synapse. Identification of the site of ADP-ribosylation as the C-terminal cysteine residue was shown using purified transducin[9]. Transducin is unusual in that it also a substrate for cholera toxin. G_o stimulates ion channels in central nervous neurons. These include non-selective monovalent cation channels, K^+ selective but non-rectifying channel and receptor triggered (eg. opioid) inhibition of presynaptic Ca^{2+} channels[40]. Muscarinic acetyl choline receptors are members of the G protein super family, five subtypes have been identified of distinct primary structure. M_2 and M_4 receptors are substrates for pertussis toxin[41] and inhibit adenylate cyclase activity. M_1, M_3 and M_5 receptors regulate inositol phosphate turnover but are not substrates for pertussis toxin. They contain a C-terminal cysteine but this is the site of palmitoylation[42].

1.2.5 Pertussis Toxin and Receptor-Mediated Signal Transduction

The G-proteins are found on the cytoplasmic surface of the plasmamembrane. The function of G proteins in receptor mediated signal transduction[43,40] is depicted in Figure 1.2. An agonist (eg adrenalin, acetylcholine, muscuranic acid) binds to its receptor and produces a change in the receptor-G protein interaction, allowing GTP to displace GDP on the α subunit. The activated GTP-α subunit has a low affinity for the $\beta\gamma$ subunits and dissociates. The free GTP-α associates with the effector (eg adenylate cyclase, K^+ channel, phospholipase C) and modifies its function. The α subunit is able to hydrolyse the bound GTP to GDP (GTPase activity) with the release of inorganic phosphate (Pi). α-GDP is released from the effector complex and re-associates with $\beta\gamma$ subunits, thus completing the cycle. Regulation of the effector targets controls the concentration of second messengers within the cell, which in turn regulate protein activity. Hence these cascade processes can control whole cell activities such as muscle relaxation and contraction, dermotaxis, exocytosis and endocytosis, energy metabolism, cell differentiation and proliferation.

Pertussis toxin catalyses the ADP-ribosylation of the α subunit, only in the presence of the $\beta\gamma$ complex[44] and the reaction is inhibited by divalent cations[45]. The ADP-ribosylated G proteins are then no longer capable of being coupled to their receptors. This is a different mechanism to that catalysed by cholera toxin, in which

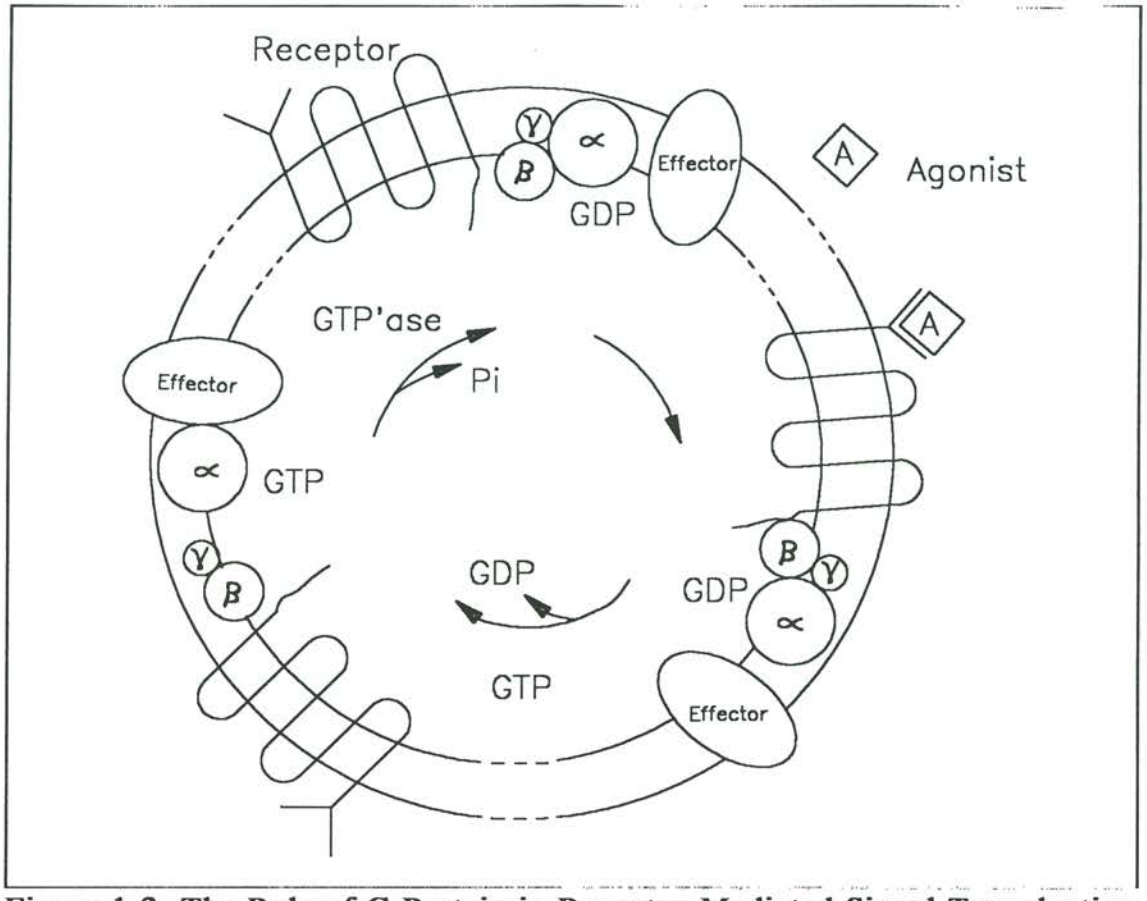


Figure 1.2 The Role of G Protein in Receptor-Mediated Signal Transduction

cytosolic ADP-ribosylating factors (ARF) and divalent cations are required for ADP-ribosylation. ADP-ribosylation of $G_s\alpha$ results in inhibition of its GTPase activity such that the α subunit becomes permanently activated, which in turn permanently activates adenylate cyclase and cAMP levels increase. Spontaneous and rapid accumulation of cAMP, independent of any receptor stimulation can occur in cholera toxin treated cells which can induce non specific effects independent of G protein function. In contrast, pertussis toxin uncouples the substrate G proteins from their receptors selectively and there is no detectable change in cAMP levels in the absence of receptor stimulation[46]. Pertussis toxin therefore, has provided a useful tool for studying the interaction of G proteins in signal transduction.

Some of the clinical effects of *B. pertussis* infection may be attributed to ADP-ribosylation of G proteins, for example, histamine sensitisation. When animals which have been pre-treated with pertussis toxin are challenged with histamine, the normal compensatory physiological response does not occur[47]. The pertussis toxin catalysed ADP-ribosylation prevents the α-adrenergic (R_i) induced release of catecholamine and

the histamine induced vasodilation is eventually fatal. Hypoglycaemia[48] and islet activating property[49] of the pertussis toxin may also be explained as a result of blockage of the α-adrenergic system which is responsible for inhibition of insulin secretion.

1.3 Endogenous ADP-ribosylation

In the early eighties Hilz and coworkers studied in detail the distribution and level of poly(ADP-ribose) and mono-ADP-ribose protein conjugates in Ehrlich ascites tumour cells[50], lymphocytes[51], developing rat liver cells[52] and rat liver subjected to various nutritional and hormonal conditions[53,54]. Several important observations were made. (1), Protein bound mono-ADP-ribose was present in several hundred fold excess over poly(ADP-ribose) modified proteins. (2), Poly(ADP-ribose) modified proteins were confined to the nucleus, whereas mono-ADP-ribosylated proteins were present in a variety of cellular compartments. (3), Mono(ADP-ribose) proteins could be further subdivided into hydroxylamine sensitive and resistant conjugates and these have different cellular locations. (4), Hydroxylamine sensitive mono-ADP-ribose conjugates were located in the endoplasmic reticulum and their levels correlated well with cell growth rates. (5), Hydroxylamine resistant conjugates were localised mainly in the mitochondria and also to some extent in the plasma membrane; their levels correlated well with hormone stimulation and cellular differentiation. In these experiments hydroxylamine sensitive conjugates refers to carboxyl ester linkages of ADP-ribose with glutamate residues; the site of modification for poly(ADP-ribosyl)ation of histone[55]. Hydroxylamine resistant conjugates included arginine cysteine and diphthamide residues[56].

In these studies cellular proteins were precipitated with trichloroacetic acid and the total amount of ADP-ribosylated protein present was estimated from the amount of radiolabel detected in the acid insoluble fractions. This technique was highly sensitive to trapping of NAD^+ which may have lead to over estimation of the total levels of ADP-ribosylated protein. However these results were confirmed, using a more sensitive technique developed by Jacobson *et al.*[57], based on fluorescent detection of released adenine containing derivatives after selective adsorption of

polymeric and monomeric ADP-ribose onto a boronate resin. Mono-ADP-ribosylated proteins have been classified according to the type of linkage between ADP-ribose and the protein. The chemical stability of glutamate-, arginine-, cysteine-, asparagine-, lysine- and diphthamide-ADP-ribose has been studied and can be used to identify the type of modification without knowing the amino-acid sequence. This is discussed in more detail in chapter five. The amount of mono-ADP-ribose modified carboxylate, arginine and cysteine present in rat liver *in vivo* have been estimated to be similar at 16, 19 and 14 pmol/mg protein respectively[58].

1.4 Endogenous mono-ADP-ribosyltransferases

The study of endogenous mono-ADP-ribosylation has been approached in two ways. The first is a direct approach where the enzymes responsible have been isolated and characterised. The properties of the endogenous ADP-ribosyltransferase, its substrates, factors which effect its activity and its distribution are all important for understanding the possible role of these enzymes in the cell. Several endogenous ADP-ribosyltransferases have been isolated to date and may be classified according to the type of ADP-ribosyl-amino acid linkage formed. These are listed in table 1.3 and will be discussed in turn. The second approach is indirect. Here the target proteins which are modified in response to external factors are identified and the cellular responses to the appearance of ADP-ribosylated protein noted. These experiments clearly suggest a role for mono-ADP-ribosylation in intracellular signalling. Some of the properties observed are listed in table 1.4 and the possible biological roles will be discussed. These whole cell studies are complex systems, and quite how mono-ADP-ribosylation interacts with the known intracellular signalling pathways is not clear. A full understanding of the mechanism may require the combination of the direct and indirect approaches in which purified ADP-ribosyltransferases are reacted with purified target proteins within a defined reconstituted system.

Table 1.3 Some Properties of Endogenous mono-ADP-ribosyltransferases

SOURCE	AMINO ACID LINKAGE	PROTEIN TARGET	EFFECT ON EUCARYOTIC CELL	REFERENCE NO.
Turkey Erythrocyte	arginine	unknown	unknown	59
Rabbit Skeletal Muscle	arginine	unknown	unknown	67
Hen Liver Nuclei	arginine	histone	Chromatin Function	71
Human Erythrocyte	cysteine	G_i	Signal Transduction	75
Hamster Kidney	diphthamide	EF 2	Inhibition of Protein Synthesis	78
Beef Liver	diphthamide	EF 2	Inhibition of Protein Synthesis	79
Rat Brain	unkown	actin	Cytoskeleton	81

1.4.1 NAD^+:Arginine ADP-ribosyltransferase

1.4.1.1 Turkey Erythrocytes

Endogenous NAD^+:arginine ADP-ribosyltransferases have been purified from a variety of sources. The first to be reported were from turkey erythrocytes[59]. A total of four ADP-ribosyltransferases designated A, B, C and D were isolated from this source[60]. Transferase A and B were soluble whereas transferase C and D were membrane associated. Transferase A was purified 544,000 fold turkey erythrocytes and had a Mr of 28,000 on SDS-polyacrylamide gels[59]. Transferase B was purified 270,000-fold and had a Mr of 32,000 on SDS-polyacrylamide gels[61]. Transferase A differed in two respects. First it was observed that the protein was converted from

an inactive oligomeric form to an active monomeric form by the addition of salt[62]. Second, Transferase A was able to use $NADP^+$ as well as NAD^+ as a substrate[63]. Like cholera toxin, transferase A and B can catalyse the transfer of ADP-ribose to a small guanidine containing compound eg agmatine. Detailed kinetic analysis of this reaction was carried out and the data was consistent with a rapid equilibrium random sequential mechanism in which the binding of the first substrate reduced the affinity of the second by more than 70 %[64]. Purified transferase A did not activate adenylate cyclase from turkey erythrocytes or any other source[65]. It did however possess ADP-ribosyltransferase activity and catalysed the transfer of ADP-ribose onto an active site arginine of glutamine synthetase[66].

1.4.1.2 Skeletal Muscle

ADP-ribosyltransferase activity was detected in sarcoplasmic reticulum from pig and rabbit skeletal muscle and sarcolemma from pig skeletal muscle[67] using small guanidine containing compounds as acceptors for ADP-ribose[68]. The enzyme responsible is an integral membrane protein and is stimulated by detergents, nucleotides, thiol reagents and magnesium ions. ADP-ribosylation of a number of acceptor proteins in skeletal muscle was noted. In the sarcoplasmic reticulum ADP-ribosylation of 100-110 kDa and 63-67 kDa proteins was observed. In the sarcolemma several proteins were labeled 205 kDa, 110 kDa (major band), 63 kDa, 54 kDa, 38 kDa and 27 kDa. Two proteins were also labelled in the glycogen pellet 83-85 kDa and 63-67 kDa. No direct correlation between the arginine specific ADP-ribosyltransferase identified and the ADP-ribosylation of the proteins observed was made. However evidence that all of the above modifications were due to mono-ADP-ribosylation of an arginine residue came from the fact that the labelling was inhibited by the presence of L-arginine methylester (an alternative substrate); the labelling was sensitive to hydroxylamine treatment and 5' AMP was released from the protein after treatment with phosphodiesterase[69]. Later experiments showed that the 100 kDa protein may be a subunit of Ca^{2+} ATPase (this is discussed in section 1.5.3).

Recently this membrane associated arginine specific ADP-ribosyltransferase was purified 215,000-fold from rabbit skeletal muscle[70] and tryptic peptides were purified and characterised. The amino acid sequences obtained were then used to

generate an oligonucleotide primer which was used in a polymerase chain reaction (PCR) based procedure to generate cDNA. A probe was designed from the PCR sequence and used to screen the rabbit skeletal muscle cDNA sequence library. A molecular clone was identified and expressed in *E. coli* but ADP-ribosyltransferase activity was not shown. The sequence obtained was novel and did not share any sequence identity with the known bacterial protein toxins. This is the first and only eucaryotic NAD^+: arginine ADP-ribosyltransferase to be characterised at the molecular level.

1.4.1.3 Hen Liver Nuclei

An NAD^+: arginine ADP-ribosyltransferase was purified from hen liver nuclei 50,000 fold[71]. The purified protein appeared as a 27.5 kDa protein on SDS-polyacrylamide gels. Agmatine, arginine, histones and casein could all serve as acceptors for ADP-ribose. The enzyme activity was inhibited by phosphate and chloride ions and did not require DNA. The ADP-ribose-histone adducts formed by the enzyme served as initiators for poly(ADP-ribosyl)ation. Phosphorylation of histones by c AMP-dependent protein kinase is inhibited by ADP-ribosylation[72]. This may be explained by the fact that the site of ADP-ribosylation (arg-34) is close to the site of phosphorylation (ser-38)[73]. The ability of the ADP-ribosyltransferase to inhibit phosphorylation of other proteins was investigated and was demonstrated for phosphorylase kinase[74]. ADP-ribosylation of purified phosphorylase kinase by the purified ADP-ribosyltransferase was achieved and subsequently shown to inhibit incorporation of phosphate onto phosphorylase kinase. The physiological relevance of these experiments is debatable, because the ratios of protein substrate, NAD^+ and enzyme used were non-physiological, and the substrate protein is a cytosolic protein and therefore is unlikely to come into contact with a nuclear enzyme.

1.4.2 NAD^+: Cysteine ADP-ribosyltransferase

A NAD^+: cysteine ADP-ribosyltransferase activity was purified from human erythrocytes 35,000-fold and appeared as a single band 28,500 Da on SDS-polyacrylamide gels[75]. Like pertussis toxin the enzyme was able to catalyse the transfer of ADP-ribose from NAD^+ to free cysteine; K_m values of 65 μM and 4.4 mM

for NAD^+ and cysteine respectively were determined. Detailed kinetic analysis of this reaction suggested that a sequential, rapid equilibrium random order mechanism was followed, similar to that of the NAD^+: arginine ADP-ribosyltransferase[64] described in section 1.4.1.1. Protein ADP-ribosyltransferase activity was demonstrated using inside out erythrocyte sealed membranes[76]. Labelling of a 41 kDa protein identical to that labelled by pertussis toxin was observed. Pre-incubation of the membranes with NAD^+ and pertussis toxin inhibited the amount of labelling observed with the purified endogenous enzyme, suggesting that the sites of ADP-ribosylation were mutually exclusive, if not identical. The chemical stability of the endogenous labelling and that catalysed by pertussis toxin were similar, ie they were both resistant to hydroxylamine. The possible biological relevance of this endogenous activity was demonstrated in platelet membranes[77]. Adrenalin, an α_2-adrenergic receptor agonist, inhibits adenylate cyclase activity in platelet membranes. Prior treatment of the membranes with [^{32}P]NAD^+ and purified enzyme lead to labelling of 41 kDa membrane protein and attenuation of the receptor mediated inhibition of adenylate cyclase.

1.4.3 Other Endogenous ADP-ribosyltransferases

Mono-ADP-ribosyltransferases which modify elongation factor-2 (EF-2) have been isolated from polyoma virus-transformed baby hamster kidney[78] and normal tissue from beef liver[79]. The endogenous enzyme catalysed reaction is much slower than diphtheria toxin fragment A, but the extent of ADP-ribosylation was similar. The endogenous enzyme was inhibited by histamine and antibodies raised against the protein identified two proteins 80 kDa and 100 kDa by immuno-blotting.

In rat brain four major polypeptides are ADP-ribosylated by endogenous ADP-ribosyltransferases corresponding to 20, 42, 45 and 50 kDa proteins on SDS polyacrylamide gels[80]. Four ADP-ribosyltransferase activities, of which one catalysed the ADP-ribosylation of actin, were partially purified from rat brain by separation on carboxymethyl-sepharose and concanavulin A columns[81]. ADP-ribosylation of actin was demonstrated using a two dimensional gel electrophoresis system which clearly resolved actin from $G_s\alpha$ subunit. The type of linkage or the factors which affect the ADP-ribosylation reaction have not been determined.

1.5 Biological role for mono-ADP-ribosylation

Evidence for mono-ADP-ribosylation of a variety of cellular target proteins in a variety of tissues is observed, suggesting a ubiquitous role in cellular metabolism. Not all of the target proteins have been identified. It is not clear whether all of the modifications reported require an ADP-ribosyltransferase to catalyse the reaction, or if they may proceed non-enzymatically.

If ADP-ribosylation is to play a role in cellular control mechanisms then it may be expected that the ADP-ribosylation modification will be reversible. As will be discussed in the following sections, the levels of ADP-ribose modified protein can vary in response to a stimulus. Assuming that the levels of target protein are unchanged then a reversible modification process is suggested. This mechanism has been demonstrated in a prokaryotic system which is described in section 1.5.1. Further evidence that such a system occurs in eucaryotes is mounting, as enzymes which can hydrolyse ADP-ribosyl-arginine[82] and ADP-ribosyl-cysteine[83] have been partially purified.

1.5.1 Reversible ADP-ribosylation Control Mechanism

The conversion of atmospheric nitrogen to ammonia by nitrogen fixing organisms is catalysed by the nitrogenase complex, which consists of two electron transferring proteins, dinitrogenase and dinitrogenase reductase. Reduction of N_2 to ammonia is an energy demanding process for which 20-30 ATP molecules are hydrolysed per N_2 reduced. It is therefore essential that the organism has an efficient control mechanism to regulate nitrogenase activity.

Kanemoto and Ludden[84] correlated the loss of whole cell nitrogenase activity in *Rhodospirillum rubrum*, upon addition of ammonia with the covalent modification of dinitrogenase reductase. Dinitrogen reductase activity is inhibited by the ADP-ribosylation of an arginine residue situated three residues away from an iron-sulphur centre, which blocks electron transfer[85]. ADP-ribosylation of dinitrogen reductase is

Table 1.4 Evidence for Mono-ADP-ribosylation Control Mechanisms

SOURCE	TARGET PROTEIN	EFFECT ON EUCARYOTIC CELL	EFFECTORS
Thyroid	G_s	Receptor-Mediated signal transduction	TSH
Liver	G_s	Receptor-Mediated signal transduction	Isoproterenol
Islets of Langerhans	G_i	Receptor-Mediated signal transduction	Adrenalin
Rod outer segments of the eye	Transducin	Receptor-Mediated signal transduction	Nitric Oxide
Adipocyte	70, 65, 61 and 52kDa plasma-membrane proteins	Unknown	Extra-cellular Calcium
Skeletal Muscle	Ca^{2+} dependent ATPase	Ca^{2+} release	Unknown
Mitochondria	38 kDa inner membrane protein and 55 kDa matrix protein	Ca^{2+} release	Unknown
Renal brush border	62 kDa membrane protein	Na^{+} dependent phosphate transport	Unknown
Brain	B-50/GAP-43	Growth and differentiation	Unknown
Platelets	GAPDH	Regulate Metabolism	Nitric Oxide
Hepatoma	78 kDa Glucose regulated protein	Regulate Metabolism	Glucose or tryptophan starvation

catalysed by dinitrogenase ADP-ribosyltransferase (DRAT). Dinitrogenase is activated by a dinitrogenase activating glycohydrolase (DRAG) which hydrolyses the ADP-ribosyl-dinitrogenase linkage[86]. The genes encoding these modulating proteins, draG and draT were identified and expressed in *Klebsiella pneumoniae*[87], an enteric N fixing organism which does not normally express this ammonium switching mechanism. Expression of draT and draG genes in *K. pneumoniae* allowed reversible ADP-ribosylation of dinitrogenase to be correlated with inhibition of nitrogenase activity in response to ammonia. These experiments provide direct evidence that reversible ADP-ribosylation is a valid control mechanism *in vivo*.

1.5.2 ADP-ribosylation and Receptor-Mediated Signal Transduction

1.5.2.1 ADP-ribosylation of stimulatory component of adenylate cyclase

Evidence that G_s function is modified by endogenous ADP-ribosylation, and is not unique to the action of cholera toxin is supported by work with rat hepatocytes and cultured bovine thyroid cells. In these systems changes in the level of ADP-ribosylation were observed in response to hormones, and endogenous ADP-ribosylation of G_s could be correlated with the activation of adenylate cyclase. A cholera-like endogenous activity was found in rat hepatocytes, in which a 55 kDa plasma membrane protein was ADP-ribosylated[88]. Endogenous ADP-ribosylation of this 55 kDa acceptor was stimulated by the action of the hormone isoproterenol in a concentration dependent manner, and adenylate cyclase activity was also stimulated. The effects were inhibited by the ß-adrenergic antagonist popranolol, suggesting that the 55 kDa protein was the stimulatory regulatory subunit of the adenylate cyclase system, G_s.

Thyrotropin (TSH) regulation of thyroid function is mediated via adenylate cyclase stimulation. The properties of TSH stimulation of cultured bovine thyroid cells was investigated[89]. ADP-ribosylation of a 40 kDa membrane protein was observed after stimulation by TSH, which appeared to be identical to the major ADP-ribosylated protein in the presence of cholera on SDS polyacrylamide gels. Like cholera toxin the endogenous enzyme was able to use arginine methylester as an acceptor for ADP-ribose. The kinetics of ADP-ribosyltransfer to the membrane protein and artificial acceptor were compared and found to be similar[90]. Both

reactions had a low affinity for NAD, K_m of 0.4 mM and pH optima pH 7-7.5. The similarity of the kinetics of each reaction suggest that they are catalysed by the same enzyme. Purification of the ADP-ribosyltransferase was not achieved. Arginine specific ADP-ribosyltransferase which have been purified from turkey erythrocytes did not ADP-ribosylate G_s (section 1.4.1.1.), which perhaps questions the validity of assuming that ADP-ribosylation of G_s and artificial substrates in crude membrane fractions are linked.

1.5.2.2 ADP-ribosylation of the Inhibitory Component of Adenylate Cyclase

There are only a few reports of endogenous ADP-ribosylation of cysteine residues in the literature. It was only recently that a protocol for identifying ADP-ribose-cysteine-protein was available[58]. Some of the early reports of endogenously labelled proteins which were found to be resistant to hydroxylamine may have been due to mono-ADP-ribosylation of cysteine residues. Several groups have observed endogenous pertussis-toxin like activity. Rat islets of Langerhans cells are able to ADP-ribosylate a 41 kDa membrane which is apparently identical to a pertussis toxin labelled substrate, the inhibitory regulatory protein of adenylate cyclase, G_i[91]. Endogenous ADP-ribosylation of G_i in human erythrocytes has been well characterised (section 1.5.2.). Endogenous enzymes which catalyse the addition[75] and removal[77, 83] of the ADP-ribose moiety onto G_i have been isolated from this source and reversible ADP-ribosylation of G_i has been demonstrated *in vitro*. ADP-ribosylation of G_i was stimulated by α adrenergic receptor agonist, adrenalin and de-ADP-ribosylation was stimulated by micro-molar concentations of free calcium.

1.5.3 ADP-ribosylation and Calcium

The intracellular distribution of calcium and in particular the level of free Ca^{2+} are extremely important in the regulation of cellular processes eg metabolism, fertilization, exocytosis, membrane transport smooth muscle contraction, sensory perception, neuronal signalling and cell division[92]. Complex spatiotemporal patterns of calcium waves and oscillations are generated within the cell from the selective release of calcium from intracellular stores through tightly regulated calcium channels. Cells have two intracellular channels for regulating calcium release from internal

reservoirs; one under the control of the ryanodine receptor and the other regulated by the inositolphosphate receptor.

1.5.3.1 Ca^{2+}-dependent ADP-ribosylation

ADP-ribosylation of proteins has been linked in two ways with calcium. The first is a cascade response to free Ca^{2+} in which calcium can effect which proteins are ADP-ribosylated in a system. This was observed in rat adipocyte membranes[93]. In the presence of calcium and NAD^+ a 70 kDa protein was labelled, in the absence of calcium however three proteins were labelled of 65 kDa, 61 kDa and 52 kDa. The second example is where ADP-ribosylation of membrane proteins alters the level of cellular calcium.

1.5.3.2 ADP-ribosylation of Ca^{2+} ATPase

The active transport of calcium from the cytoplasm into the sarcoplasmic reticulum occurs at the expense of ATP hydrolysis through Ca^{2+} dependent ATPase. Direct modification of Ca^{2+} dependent ATPase from rabbit muscle has been reported[94], using a reconstituted system containing partially purified Ca^{2+}-dependent ATPase and ADP-ribosyl transferase purified from rabbit skeletal muscle. Modification of the 105 kDa subunit of the Ca^{2+}-dependent ATPase was observed on SDS polyacrylamide gels and could be correlated with the inhibition of its ATPase activity. These experiments suggested that ADP-ribosylation of the sarcoplasmic reticulum may play a role in the regulation of Ca^{2+} in skeletal muscle. The most pronounced effects however have been observed in rat liver mitochondria. ADP-ribosylation of two major mitochondrial proteins has been reported[95]. The first is a 50 kDa protein found in the mithochondrial matrix and the second is a 31 kDa protein located on the inner surface of the inner mitochondria membrane that has been associated with Ca^{2+} eflux from the mitochondria.

1.5.3.3 Enzymatic versus non-enzymatic ADP-ribosylation in Rat Liver Mitochondria

There is however some debate as to whether the apparently specific ADP-ribosylation of mitochondrial proteins are true ADP-ribosyltransferase reactions as the proteins could also be modified by free ADP-ribose[96]. NAD^+ concentrations in the

mitochondria are very high (1-2 mM) and NAD glycohydrolases have been located and purified from the inner surface of inner mitochondrial membrane[97]. Richter *et al.*[98] proposed that Ca^{2+} eflux from the mitochondrion in response to oxidation and hydrolysis of pyridine nucleotides was linked to ADP-ribosylation of the 31 kDa protein, a putative Ca^{2+}/H^{+} anti-porter . Evidence that ADP-ribosylation of the 31 kDa protein was catalysed by an ADP-ribosyltransferase was presented by Masmoudi and Mandel[99], who separated the NAD glycohydrolase activity away from ADP-ribosyltransferase activity contained in solubilised mitochondrial membranes by a two step chromatographic procedure.

1.5.3.4 Cyclic ADP-ribose and Ca^{2+} Mobilisation

Most recently intracellular Ca^{2+} mobilisation in cells has been linked with a cyclic intermediate of NAD^{+}[100], the structure of which has been identified as cyclic ADP-ribose[101]. Unexpectedly an enzyme generating cyclic ADP-ribose has been identified as CD38 surface antigen of human lymphocytes from comparison of the amino-acid sequence of an ADP-ribosyl cyclase from *Aplysia*[102]. Enzymatic activity of the surface antigen has since been confirmed[103]. A role for cyclic ADP-ribose as a second messenger for calcium mobilisation in the endoplasmic reticulum has been demonstrated in pancreatic ß cells[104] and synthesis of cyclic ADP-ribose is enhanced by cGMP[105]. In cardiac muscle the mechanism of action of cyclic ADP-ribose is to activate directly the non-skeletal type ryanodine receptor Ca^{2+} channel, by increasing the frequency of the opening of the channel[106]. How this is achieved has not been shown; it may be that cyclic ADP-ribose covalently modifies the ion channel and such a process may account for some of the non-enzymatic ADP-ribosyltransfer reactions reported. Cyclic ADP-ribose activates non-skeletal forms of the ryanodine sensitive Ca^{2+} channel only.

1.5.4 Nitric Oxide

The effects of nitric oxide, a short-lived highly reactive gas, on cellular regulation are widespread and diverse, e.g. tumoricidal and bactericidal properties of white blood cells, smooth muscle relaxation and neurotransmitter[107]. This small molecular effector is generated in cells by the action of nitric oxide synthetase from arginine. The reaction requires NADPH and molecular oxygen, and is under the control of Ca^{2+}/calmodulin. Nitric oxide can diffuse freely across membranes and its effects are mediated through guanylyl cyclase. Nitric oxide binds to the haem part of soluble guanylyl cyclase, stimulating the formation of cyclic GMP, which in turn stimulates protein kinase and increased phosphorylation of target proteins. Several groups have noted changes in the levels of ADP-ribosylation of specific proteins in response to nitric oxide suggesting an alternative cGMP independent cascade system.

1.5 4.1 Rod outer Segment Membranes

Several membrane proteins are labelled by endogenous ADP-ribosylation in rod outer segments of the eye, 116 kDa, 66 kDa, 46 kDa and transducin,[108]. ADP-ribosylation of the 116 kDA, 66 kDa and 46 kDa proteins were inhibited by exposure of the membranes to nitric oxide, whereas transducin was stimulated. Whether the effects observed were due to modification of the substrates or of the ADP-ribosyltransferases concerned was not addressed.

1.5.4.2 Platelets

Brüne and Lapetina[109] observed a novel endogenous ADP-ribosyltransferase activity in human platelets. The activity was stimulated by nitric oxide and lead to ADP-ribosylation of a 39 kDa cytosolic protein. Several unique characteristics of this transferase reaction were noted[110]. (1), It was stimulated by nitric oxide. (2), Thiol reagents were required for activity to be observed. (3), The reaction was inhibited by treatment with N-ethyl maleimide (NEM). (4), The reaction was stimulated by phosphate ion and inhibited by sodium chloride. (5), The modified protein was stable to hydroxylamine and mercuric chloride. (6), ADP-ribosylation was independent of guanylyl cyclase. (7), NADPH, but not $NADP^+$ could stimulate the reaction.

Characterisation of the substrate protein was important to understand the

relevance of this modification. Sufficient protein was recovered from a gel to perform N-terminal sequence analysis. Comparison with known sequences identified the protein as glyceraldehyde-3-phosphate dehydrogenase (GAPDH)[111]. It was then discovered that the reaction was non-enzymatic. GAPDH was auto-ADP-ribosylated and inactivated in the presence of [^{32}P]NAD^+ and nitric oxide releasing reagents only[112]. Analysis of the chemistry of the reaction by Mc Donald and Moss[113] showed that whole NAD^+, and not ADP-ribose was covalently bound to GAPDH. A mechanism for this was proposed in which cysteine groups react with nitric oxide. The reactive intermediate, S-nitrosylated cysteine is then able to react directly with NAD^+ and covalently modify the enzyme. The ability of other dehydrogenase enzymes to be modified was investigated. Labelling and inactivation of alcohol dehydrogenase, lactate dehydrogenase[114] and aldehyde dehydrogenase[115] have since been reported although different mechanisms are involved as they were not stimulated by nitric oxide. Although NAD^+ dependent modification of GAPDH is not a true ADP-ribosyltransferase reaction it may be important physiologically and may explain some of the toxic effects of nitric oxide.

1.5.5 Growth and Differentiation

1.5.5.1 GTP-binding Proteins

In neuronal tissue, growth is regulated by a family of growth associated phosphoproteins (GAP). GAP-43 is the best characterised of these proteins. Its expression has been closely correlated with axonal growth and it is found on the inner surface of the plasmamembrane, especially at the growth cone, where it interacts with G proteins. Although its normal action *in vivo* is unclear, GAP-43 has been shown to bind calmodulin, be posphorylated by protein kinase C and to inhibit phosphatidylinositol phosphate kinase. Most recently it has been shown to be ADP-ribosylated implicating a role for ADP-ribosylation in growth and regeneration in the nervous system[116].

1.5.5.2 Nutritional Stress

The family of 70 kDa heat shock proteins and glucose-regulated proteins are synthesised and accumulated in the cell when the cell is challenged eg starved of a vital nutrient. These proteins are involved in the assembly of secretory and integral membrane proteins. The major mono-ADP-ribose acceptor protein *in vivo* in avian and murine cells was identified as 83 kDa stress induced and glucose-regulated protein[117]. Reversible ADP-ribosylation of the 78 kDa glucose-regulated protein in mouse hepatoma cells in response to starvation of the cells with either glucose or tryptophan has been reported by Leno and Ledford[118] thus implicating ADP-ribosylation with a novel cellular metabolic control mechanism.

1.6. Aims of Project

The level of understanding of the nature of these endogenous ADP-ribosyltransferases is poor relative to the extent of knowledge concerning the bacterial toxins. Evidence supported by indirect studies show that endogenous ADP-ribosylation is an important covalent modification of proteins by which their activities are modified. Endogenous ADP-ribosyltransferase activity has been linked with a variety of cellular processes eg receptor mediated signal transduction, calcium signalling and growth and differentiation. Some of the activities appear to mirror the action of bacterial toxins while others appear to be unique to certain cell types. The ADP-ribosylation modification is quite specific compared to phosphorylation; only a few modified protiens are commonly observed ranging in molecular weight from 21 kDa to 120 kDa. Most of these proteins are membrane bound and another common feature is their ability to bind nucleotides. Relatively few endogenous ADP-ribosyltransferases have been purified and most of these have been arginine specific. *In vivo* estimates of the amount of ADP-ribosylated protein suggest that proteins modified through cysteine residues are equally abundant. However only one NAD^+: cysteine ADP-ribosyltransferase has been purified. A classical protein biochemical approach to purify a NAD^+: cysteine ADP-ribosyltransferase activity was taken in favour of a molecular biological one because of the lack of amino acid identity between the known ADP-ribosyltransferases on which to base oligonucleotide probes.

The aims of the project were:

(1) To purify an endogenous cysteine specific ADP-ribosyltransferase from bovine blood.

(2) To investigate the kinetic properties of this purified enzyme and compare them to the known endogenous ADP-ribosyltransferases and the bacterial protein toxins.

(3) To demonstrate ADP-ribosylation of a target membrane protein in re-sealed inside out erythrocyte membranes and see if it is the same as pertussis toxin.

(4) To raise antibodies against the purified ADP-ribosyltransferase such that the distribution of the enzyme could be investigated.

(5) To determine the N-terminal amino acid sequence of the purified protein to facilitate a data base search and to generate oligonucleotide primers with which to screen cDNA libraries.

METHODS

2.1 Materials

Purified pertussis toxin was a kind gift from Dr L. Irons (the public health laboratories, Porton down, Salisbury, U.K.) and also Dr K. Capiau (Smithkline Beecham, Rixensart, Belgium). Radiochemicals, [^{3}H-nicotinamide]NAD^+, [carbonyl-^{14}C]NAD^+ and [^{32}P-adenylate]NAD^+ were purchased from Amersham and [^{3}H-adenine]NAD^+ was purchased from New England Nuclear. Antibodies against the C-terminal portion of $G_i\alpha$ and $G_s\alpha$ were a kind gift from Dr G. Milligan (department of Biochemistry, Glasgow University). Horseradish peroxidase anti-mouse IgG and anti-rabbit IgG conjugates were supplied by the Scottish antibody production unit (SAPU). All other chemicals used were of the highest grade possible.

2.2 Activation of Pertussis Toxin

Activation of the whole toxin is requires before NAD^+-glcohydrolase activity or ADP-ribosyltransferase activity can be detected. Activation of the toxin is achieved by promoting the release of S1 subunit from the B oligomer and the reduction of an intramolecular disulphide bond of the S1 subunit. The optimised conditions described by Kaslow *et al.*[22] were followed. Pertussis toxin was stored as a saturated ammonium sulphate suspension (2 mg/mL), at 4°C. The pertussis toxin precipitate was removed by centrifugation (13000g, 10 min) and then resuspended in 50 mM potassium phosphate, 100 mM NaCl, pH 7.0. Excess ammonium sulphate was removed by dialysis. The toxin was incubated with 10 mM dithiothreitol, 0.1 mM ATP and 1% CHAPS at 37°C for 15 min before assaying for activity.

2.3 NAD-Glycohydrolase Assay

This may be considered to be the enzymatic half reaction of ADP-ribosyltransfer. The enzyme catalyses the hydrolysis of the glycosidic linkage between ADP-ribose and nicotinamide of NAD^+. Water or free aminoacid acts as a nucleophilic acceptor for the ADP-ribose moiety. Moss and Vaughan[119] developed an assay to follow this reaction by measuring the concomittant release of nicotinamide. Simply, ^3H nicotinamide product was separated from [^3H nicotinamide]NAD^+ using a small miniprep ion exchange column.

2.3.1 Incubation

A typical assay contained 50 mM phosphate buffer pH 7.0 and 100 μM (^3H nicotinamide)NAD^+ (1 mCi/mmole). Control incubations, without any enzyme added, were run for each reaction condition to account for background non-enzymic hydrolysis of NAD^+. Reactants were added to give a final reaction volume of 150 μL and incubated in a water bath at 30°C.

2.3.2 Detection Nicotinamide Released

The reaction was stopped by addition 0.85 mL ice cold water and then 0.9 mL aliquot removed and applied to 0.5 mL washed Dowex AG1X anion exchange resin (Sigma). Unbound [^3H]nicotinamide was washed off the column with two volumes of water 0.5 mL each. An aliquot of 0.5mL was removed from 2 mL of eluate and 4 mL of scintillation cocktail (HiSafe, Pharmacia/ Ultima Gold, Packard) was added. Radioactivity was detected by scintillation counting for 2-10 minutes using a Packard 1900CA scintillation counter. Duplicate assays were measured for each time point and reaction conditions set such that a maximum of 20% NAD^+ was turned over. Pertussis toxin catalysed NAD'ase activity ($pmol^{-1}min^{-1}$) was calculated using:

$$\frac{\text{[Average counts released(PT) - Background] x dilution factor (cpm)}}{\text{Specific Activity } NAD^+\text{(cpm/pmol) x Time (min)}}$$

Similarly, the cysteine dependent activity of the endogenous enzyme was

calculated. Instead of total counts detected the difference between incubations with and without cysteine for enzyme and blank was determined.

$$\frac{[\text{ENZYME}(\text{cpm}(+\text{cys}) - \text{cpm}(-\text{cys})) - \text{BLANK}(\text{cpm}(+\text{cys}) - \text{cpm}(-\text{cys}))]\ (\text{cpm})}{\text{Specific Activity } NAD^+(\text{cpm/pmol}) \times \text{Time (min)}}$$

2.3.3 Curve Fitting

The rates of nicotinamide release, calculated under various assay conditions, were used to determine kinetic parameters for pertussis toxin and the purified cysteine dependent NAD glycohydrolase (see chapter four). A non-linear regression curve fitting package was used to fit the data to proposed kinetic models. The package used was Regression (Blackwell Scientific Publications Ltd) and was operated under a Windows environment. Non-linear regression was used in preference to linear transformations of the data which can distort experimental results. The parameters for a line are optimised iteratively and by minimising the sum of the squares, thus giving the "goodness of fit" of a data set for a particular model. It does not find a best fitting line through a data set. Simple weighting was used unless otherwise stated.

2.4 The Separation of $NAD^+$and ADP-ribose by HPLC.

2.4.1 Reverse Phase HPLC

The products of the NAD-glycohydrolase assay were separated out on an ODS-HYP 2734 (HIGHCHROM) reverse-phase column (250 mm x 4.6 mm) using ALTEX equipment, following the methodology developed by Lobban and van Heyningen[120]. The relative retention times of the charged nucleotides on the reverse-phase support are maximised by the use of a quarternary ammonium "solvophobic ion".

The column was stored in 20 % isopropanol, and was cleaned by washing with one column volume 100 % isopropanol (HPLC grade, BDH) and then rinsed with filtered, de-gassed, double distilled water (five column volumes, 0.1 mL/ min, maximum pressure 3000 p.s.i.). The column was equilibrated with 95% 10 mM ammonium phosphate buffer pH 3.5, 5% methanol (filtered and de-gassed). The

optimum running conditions, for separation of the products was 1 mL/min, 1000 p.s.i.

2.4.2 Ion Exchange HPLC

With time the reverse phase column deteriorated. This may have been compounded by the use of ammonium salts which are known to shorten column life by irreversible changes of the stationary phase characteristics. Slow flow rates had to be employed to avoid high pressures, which effected the retention time of ADP-ribose such that resolution of potential ADP-ribose-cysteine and ADP-ribose could not be achieved. An alternative separation method was employed which separated the products of the NAD-glycohydrolase assay on the basis of charge using a Sherisorb-SAX ion exchange column (50 x 4.6 mm) (Anachem). The method of West[9] was followed. The column was stored in 25 % methanol and was prepared for use by consecutive washing with one column volume of double distilled water, one column volume of 1 M NaCl and one column volume of double distilled water. The column was equilibrated with ten column volumes of 100 mM mono-basic potassium phosphate pH 4.5. Separation of the products of NAD-glycohydrolase reaction was achieved by isocratic elution at a flow rate 1 mL/min, 1000 psi. All reagents were filtered and de-gassed before applying to the column.

2.4.3 Sample Treatment

After the NAD-glycohydrolase incubation was complete protein was removed from the assay mix by filtration through a membrane with a cut off of *Mr* 10,000 (Millipore, micro-concentrator). The filtrate was stored on ice and then 100 μL aliquot was removed and analyzed by HPLC. The products of the NAD glycohydrolase assay were eluted isocratically and detected by a U.V. monitor at 254 nm. Fractions were collected at 15 second time intervals using an LKB fraction collector. 4 mL of scintillation fluid (HiSafe, Pharmacia/ Ultima Gold, Packard) was added and counted in Packard 1900 CA scintillation counter for 10 minutes to detect [^{3}H]ADP-ribose label. NAD^+, ADP-ribose, AMP, ADP and ATP were run routinely as standards.

2.5 Protein Analysis

2.5.1 Protein assays

The concentration of soluble protein was estimated using the Bradford reagent[121]. A five times concentrated stock solution was diluted to a one times working solution as required. 100 μL of protein sample was mixed with 900 μL diluted Bradford reagent and a blue colour was allowed to develop for a minimum of 15 min at room temperature. The absorbance at 595 nm was measured using a Thecil spectrophotometer. The protein concentration was estimated from a standard curve generated using a 1 mg/mL bovine serum albumin stock solution. The reaction was linear over the range of 1 - 25 μg protein per mL of reagent.

The concentration of membrane proteins was estimated using Peterson's[122] simplification of the method of Lowry[123]. 5-60 μg of protein was precipitated at room temperature on addition of 0.15% (w/v) sodium deoxycholate and 10% (w/v) TCA. The precipitate was collected by centrifugation (10,000 g, 5 min). The pellet was solubilised in copper tartrate carbonate solution containing 2.5% SDS. The protein was then mixed with Folin-Ciocalteau phenol reagent and the absorbance at 750 nm was measured using a spectrophotometer (Thecil) after 30 min. BSA was used as a standard for this reaction.

2.5.2 SDS Polyacrylamide gel electrophoresis

Proteins were separated under denaturing conditions using discontinuous buffer system[124]. Tall Mighty Small mini-gel apparatus was used (Hoefer Scientific Instruments). There are several advantages of this system, compared to the large slab gel apparatus. Less reagents are required (smaller quantities of acrylamide - a neurotoxin - have to be handled). The process is quicker due to faster separation times (90 min compared to overnight) and faster staining procedures (15 min compared to several hours with Coomassie Blue). Also, less sample is required, as little as 1 μg protein/band is detectible by Coomassie staining, using this system.

2.5.2.1 Preparation of gels

The resolving gel mix contained 0.375 M Tris-HCl, pH8.8, 0.1% Sodium dodecyl sulphate (SDS) and a stock solution of 30% (w/v) acrylamide, 0.8% (w/v) bis-acrylamide was added to give the required percentage of acrylamide for resolution of proteins. Pertussis toxin sub-units were routinely separated on 17½% acrylamide gels. The purification of cysteine dependent NAD'ase was monitored using 12% gels and ADP-ribosylated membrane proteins were separated on 10% gels. Polymerisation was initiated by the addition of freshly prepared 10% (w/v) ammonium per sulphate (APS) and TEMED to give a final concentration of 0.1% (w/v) and 0.07% (v/v) respectively. A 4% (w/v) acrylamide stacking gel was applied to the top of the resolving gel containing, 0.125 M Tris-HCl, pH 6.7, 0.1% (w/v) SDS, 0.05% (w/v) APS and 0.05% (v/v) TEMED.

2.5.2.2 Sample Treatment

A four times concentrated sample buffer was prepared containing, 0.25 M Tris-HCl pH6.8, 8% SDS, 40% glycerol, 20% (v/v) 2-mercaptoethanol and 0.1% (w/v) bromophenol blue. Soluble samples were dialysed against low ionic strength buffer to remove salt and sample buffer was added one part to three. The protein samples were then placed in a boiling water bath for 2 min, cooled and then spun 13000 g to remove any aggregates. 0 - 20 μg of protein in 30 μL was loaded per lane. Insoluble precipitated protein samples were solubilised in 8 M urea (freshly prepared) first, and then sample buffer was added as described above. Heating was not necessary to solubilise proteins treated in this way. Membrane proteins were solubilised in 50 mM Tris-HCl, pH 6.8, 2% SDS by heating 85°C for 20 min and then treated as described for the soluble sample.

2.5.2.3 Coomassie Staining

0.25 g Coomassie Brilliant Blue R was dissolved in 1 L 40% Methanol, 7% acetic acid. This solution fixes and stains the proteins in the gel. Gels were stained for 15 - 60 min and destained 60 - 180 min with 5% methanol, 7% acetic acid. Sponges were added to the destain solution to bind the excess coomassie dye. As little as 1 μg protein/band is detectible by this staining procedure.

2.5.2.4 Silver Staining

A modification of the staining procedure described by Wraight[125] was followed. Gels were fixed for 15 min in methanol: acetic acid: water (20:10:7 (v/v)). The gels were washed three times in 50 % methanol for a minimum of 1 h each. Between washes the gel was re-hydrated with distilled water. 0.4 g silver nitrate was disolved in 2 mL distilled water and added dropwise to 21 mL 0.1 M sodium hydroxide, 1M ammonia solution and then diluted to 100 mL. The gel was stained for 15 min. The gel was washed with distilled water three times (2 min each) and then developed using 0.005% (w/v) citric acid, 0.005% (v/v) formaldehyde developer. The reaction was stopped by addition methanol: acetic acid: stop solution. Siver stain and developer were prepared fresh on the day of use. Some gels were stained by both procedures. Staining by one method does not affect the other.

2.5.3 Western Blotting

Specific proteins separated out on SDS polyacrylamide gels could be identified with an antibody probe using the Western blotting technique. First, the proteins are transfered from the gel onto a nitro-cellulose membrane. This is achieved by constructing a "gel sandwich" consisting of three layers of blotting paper (Whatmann 3 MM) soaked in blotting buffer (50 mM potassium phosphate, 20% methanol, pH 7.4), the gel, the nitro-cellulose membrane and three more sheets of buffer soaked blotting paper. The "gel sandwich" is clamped tightly together and immersed in blotting buffer. A voltage is then applied across the "gel sandwich". The negatively charged proteins move towards the positively charged terminal but are immobilised onto the nitro-cellulose. Complete transfer of erythrocyte ghost proteins from a 10% polyacrylamide gel was achieved using transfer conditions of 1 A, constant current for 2 h.

Excess protein binding sites on the nitro-cellulose are blocked for 1 h by incubation with 1% (w/v) dried milk solids (Marvel) in 0.1% (v/v) TWEEN in Tris buffered saline, 50 mM Tris HCl, 150 mM NaCl, pH 7.4 (TBST). The blot is then probed with the antibody raised against the protein of interest, eg anti-$G_i\alpha$ and anti-$G_s\alpha$ (see chapter five, section 5.2.2). These antibodies were diluted 1/2000 with TBST and were incubated with the blot for 2 h at room temperature. The unbound

and loosely associated antibody were removed by sequential washing of the blot, three times, with TBS, and TBST for 5 min each. Horseradish peroxidase IgG conjugate was diluted 1/4000 to probe the blot for 1 h. The IgG component recognises the first antibody and the catalytic activity of horseradish peroxidase is used to detect the complex by the formation of a luminescent compound. Unbound and loosely associated second antibody were removed by the sequential washing procedure described above. ECL reagents (Amersham) are added to the washed blot and then exposed to hyperfilm MP autoradiography film for 1-10 min. The ECL reagents are used up after 2 h and the antibody can nolonger be detected. The autoradiography film is developed using an automatic X-ray film developer.

2.5.4 Electro-elution of Protein from Polyacrlamide Gels

Strips of polyacrylamide gel containing protein were removed from the gel and cut into 10 mm by 2 mm portions. These were positioned above the concentrating cup on a piece of nylon membrane in the ISCO electroelution apparatus. The buffer used was 10 mM potassium phosphate pH 7.5, 0.1% SDS. The proteins were eluted on application of a constant voltage of 100 V for 3 h. The negatively charged protein moves out of the gel matrix towards the cathode. The progress of the protein is halted by the presence of a dialysis membrane of relative molecular weight cut-off of 5000, which seals the bottom of the concentrating cup. The eluted protein is concentrated in the concentrating cup in a total volume of 50 μL.

PURIFICATION OF AN ENDOGENOUS MONO-ADP-RIBOSYLTRANSFERASE FROM BOVINE ERYTHROCYTES

3.1 Introduction

Mono-ADP-ribosylation is a covalent modification of proteins in which a single ADP-ribose is transferred from NAD^+ to an acceptor protein with concomitant release of nicotinamide and a proton. A classic example of such a reaction is exhibited by bacterial protein toxins. Each toxin modifies a specific amino-acid for a specific target protein, e.g. cholera toxin ADP-ribosylates arg-201 of $G_s\alpha$[126], pertussis toxin modifies C-terminal cys-352 residue of $G_i\alpha$[9] and diphtheria toxin labels the modified amino acid residue diphthamide of elongation factor 2[3]. Such modifications have a critical role in the pathogenesis of disease, by blocking of signal transduction pathways or switching off protein synthesis. However ADP-ribosylation also occurs physiologically; these reactions are less well characterised. For a detailed review see chapter one.

Endogenous ADP-ribosylation has been studied by direct and indirect approaches. The direct approach identifies the mono-ADP-ribosyltransferases involved and can be used to answer questions such as: What is their substrate specificity? What is their distribution? What factors effect their activity? Endogenous ADP-ribosyltransferase activity showing the same specificity as bacterial protein toxins has been identified in a variety of cell types. The enzymes responsible have rarely been purified. The notable exceptions are the NAD^+: arginine-ADP-ribosyltransferases from turkey erythrocytes[59] and rabbit skeletal muscle[71] and NAD^+: cysteine ADP-ribosyltransferase from human erythrocytes[76, 77].

Indirect evidence comes from the study of the target proteins and the factors which influence the level of ADP-ribosylation observed. These experiments hint towards the physiological role of mono-ADP-ribosylation. Measurement of the level of ADP-ribosylated protein *in vivo* shows that mono-ADP-ribosylation occurs mainly outside the nucleus and accounts for 90 % of all ADP-ribosylated protein[127]. A role

in signal transduction has been suggested, as levels of ADP-ribosylation are sensitive to cAMP dependent[128] and independent pathways[108]. Mono-ADP-ribosylation of target proteins has been shown to cause Ca^{2+} flux[93], phosphate transport[129] and loss of target enzyme activity[74, 114].

The aim of this project was to take a direct approach, to purify and characterise an endogenous ADP-ribosyltransferase from bovine erythrocytes, which had a similar substrate specificity to pertussis toxin. Bovine erythrocytes were chosen as a source for the enzyme because large volumes were regularly obtainable from the slaughter house and were relatively safe to handle. To purify the enzyme it was assumed that, like pertussis toxin, it would catalyse the transfer of ADP-ribose to $G_i\alpha$ and also have a cysteine dependent NAD^+ glycohydrolase (NAD'ase) activity. The NAD'ase reaction describes the hydrolysis of NAD^+ to ADP-ribose, nicotinamide and H^+ and can be measured by detecting the amount of [^{3}H]-nicotinamide released from [^{3}H-nicotinamide]NAD^+ by separation of the product on an ion exchange resin. The role of cysteine in the assay would be to act as an acceptor for ADP-ribose or to activate the enzyme resulting in an increase of the rate of nicotinamide released. Cysteine dependent activity is measured as the difference between duplicate assays with and without cysteine. This assay was adopted to monitor the progress of the purification procedure because it allows a large number of samples to be screened at one time; it is quantitative; it is quick and relatively cheap. The ADP-ribosyltransfer assay is impractical because only a limited number of samples can be assayed at one time; it is difficult to quantitate; it is time consuming and expensive.

In this chapter the development of a three step purification to purify a cysteine dependent NAD'ase is described. Three major problems had to be addressed. (1) The major contaminant from which it is purified is one protein, haemoglobin, which probably accounts for more than 95 % of the total soluble protein in the bovine erythrocyte. (2) A variety of NAD'ase and ADP-ribosyltransferase activities are present in the crude fractions from which the cysteine dependent activity has to be resolved. (3) The cysteine dependent activity was extremely labile.

3.2 Preparation of Bovine Erythrocyte Lysate

3.2.1 Method

1 L bovine blood was collected (J. Stoddart Ltd, Slateford slaughter house, Edinburgh) and 0.1 L 3.8 % (w/v) sodium citrate was added as an anti-coagulant. The erythrocytes were centrifuged (5000g, 10 min) and washed three times with phosphate-buffered saline, 12.7 mM potassium phosphate, 133 mM NaCl, 3 mM KCl, 1 mM $MgCl_2$, pH 7.4 (PBS). The supernatant and the top layer of cells were discarded for each wash to ensure efficient removal of platelets. The cells were lysed with 10 mM sodium phosphate, 0.1 mM phenylmethylsulphonyl fluoride (PMSF), 0.1% 2-mercaptoethanol, pH 7.8 hypotonic buffer. Unbroken cells and ghost membranes were removed by centrifugation (14000g, 30 min).

3.2.2 Results

It was estimated that 1 L of blood produced 60-80g protein using Bradford reagent with BSA as a standard (as described section 2.5.1). This was a lower estimate than expected, as normal cows have 115 g/L haemoglobin in their blood. If the Bradford reagent does not react with haemoglobin to the same extent as with BSA, it could cause significant lowering of the protein estimation, since haemagloblin was the major protein present in this fraction. Alternatively, this low protein yield may be explained by the washing procedure used to prepare the bovine erythrocytes. The erythrocytes were washed three times with PBS. After each wash the top layer of cells was discarded to ensure efficient removal of platelets, which appear as a shiny layer of cells between the loose erythrocyte pellet and the buffy coat. The lysate contained a high level of non specific NAD'ase activity. Figure 3.1 shows the dose response curve obtained for fresh lysate in the presence and absence of 100 mM cysteine. A small increase in the rate of nicotinamide released was observed in the presence of cysteine and this difference was measured as the cysteine dependent NAD'ase. The rate of nicotinamide release in response to addition of greater than 2.5 mg lysate proteins in the presence of 100 mM cysteine became non-linear. This was probably due to substrate depletion and/or product inhibition, since 30% of the total NAD^+ (15 nmole) is hydrolysed during the time course of the assay (30 min) on

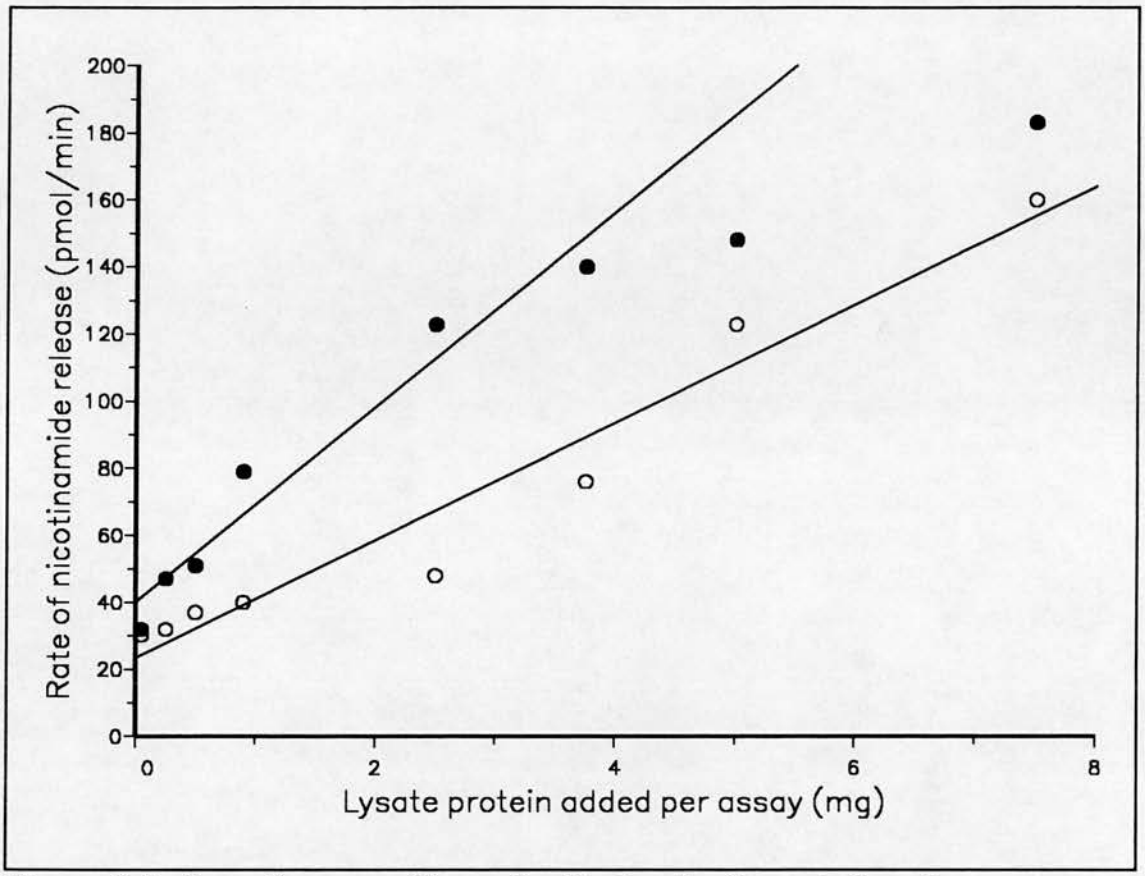


Figure 3.1 Dose Response Curve for Bovine Erythrocyte Lysate
Duplicate assays, with [●] and without [○] 100 mM cysteine were set up and the NAD'ase assay was performed as described in section 2.3.

addition of 2.5 mg lysate proteins. The rate of cysteine independent NAD'ase activity was linear over the range of lysate protein added (0 - 7.5 mg). A cysteine dependent increase in the rate of nicotinamide release was only observed in freshly prepared lysate and was not detectible after storage at 4°C overnight. To detect the cysteine dependent activity reliably, it must constitute more than 15% of the total NAD'ase activity in the presence of cysteine, in order that any significant difference between assays with and without cysteine can be measured. From the linear portion of the dose response curve the specific activities of the lysate protein were calulated. Total NAD'ase activity in the absence of cysteine is 60 pmol/min/mg. The addition of 100 mM cysteine increases NAD'ase activity observed 100%, to 120 pmol/min/mg. Calculating the difference between these activities gives a cysteine dependent activity of 60 pmol/min/mg.

3.3 Ammonium Sulphate precipitation

Ammonium sulphate precipitation provides a crude fractionation step which can easily be applied to large sample volumes (1 L). Different proteins have different charges and will be selectively salted out at different concentrations of ammonium sulphate. As a result very dilute components of the protein mixture may be concentrated and clearly separated from the bulk of the contaminating protein.

3.3.1 Method

A subtractive ammonium sulphate cut was performed on the crude lysate at 4°C. Solid ammonium sulphate (BDH, especially low in heavy metals) was added to give a 35% saturated ammonium sulphate solution (at 20°C). The precipitate was removed by centrifugation (14,000 g, 30 min) and the supernatant was increased to 40% saturated ammonium sulphate solution. This procedure was repeated from 35% to 80% of saturation in 5% intervals. The protein precipitated in each fraction was solubilised in 50 mM phosphate buffer, pH 7.0, 30% ethylene glycol, 0.1 mM PMSF, 0.1 mM EDTA and 1.0 mM benzamidine (buffer A). Each fraction was assayed for cysteine dependent NAD'ase activity (section 2.3) and for the amount of protein present (section 2.5.2).

3.3.2 Results

Table 3.1 shows the data obtained using this subtractive cut procedure. NAD'ase activity was precipitated at low ammonium sulphate saturation and was clearly separated from the bulk of the protein. Cysteine dependent activity was concentrated in the 35 - 45% saturation fractions. 25 fold purification was ahieved, as the bulk of the haemoglobin was removed. Gels (not shown) of the proteins precipitated between 30 and 60 % saturated ammonium sulphate showed several bands in the region of 20 - 80 kDa and some haemoglobin. At 60% of saturation and above only haemoglobin was evident.

Table 3.1 Subtractive Ammonium Sulphate Cut of Bovine Erythrocyte Lysate

% OF SATURATION AMMONIUM SULPHATE	Total Protein (mg)	TOTAL ACTIVITY (nmol/min)	SPECIFIC ACTIVITY (pmol/min/mg)
0	75500	4500	60
30	2400	-	-
30-40	1800	3000	1660
40-45	2700	1200	440
45-50	11600	-	-
50-60	27500	-	-
60-70	30500	-	-
70-80	-	-	-

Following this experiment, precipitation with 40% Ammonium sulphate saturation was adopted as the first step of the purification. 80% of the protien was removed by this step, but was still contaminated by non-specific NAD'ase activity the dose response curve in the presence and absence of cysteine is shown in Figure 3.2. Activity yields of 70 - 90% were routinely achieved from fresh material. The stability of the cysteine dependent activity, stored as an ammonium sulphate precipitate under various conditions was tested. Figure 3.3 shows that the activity was extremely labile at 4°C but was stable on freezing at -16°C. No loss in activity was seen over three months.

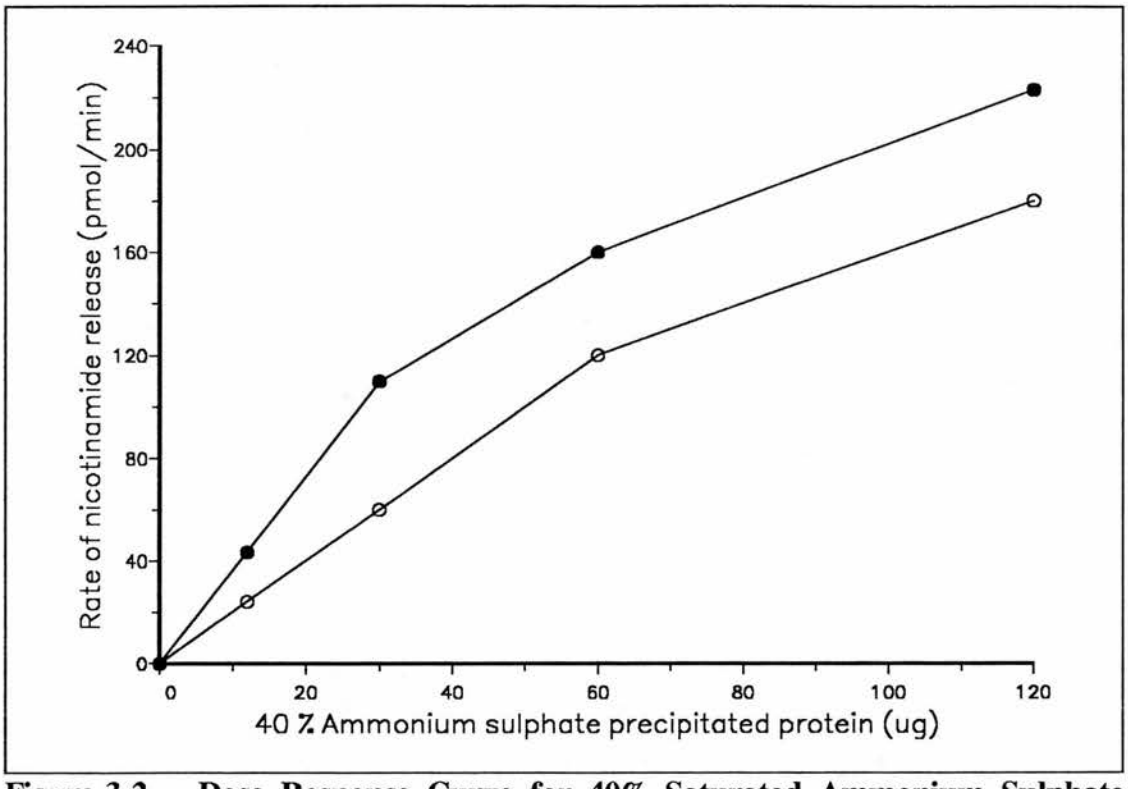


Figure 3.2 Dose Response Curve for 40% Saturated Ammonium Sulphate Fraction. Duplicate assays, with [●] and without [○] 100 mM cysteine were performed as described in section 2.3.

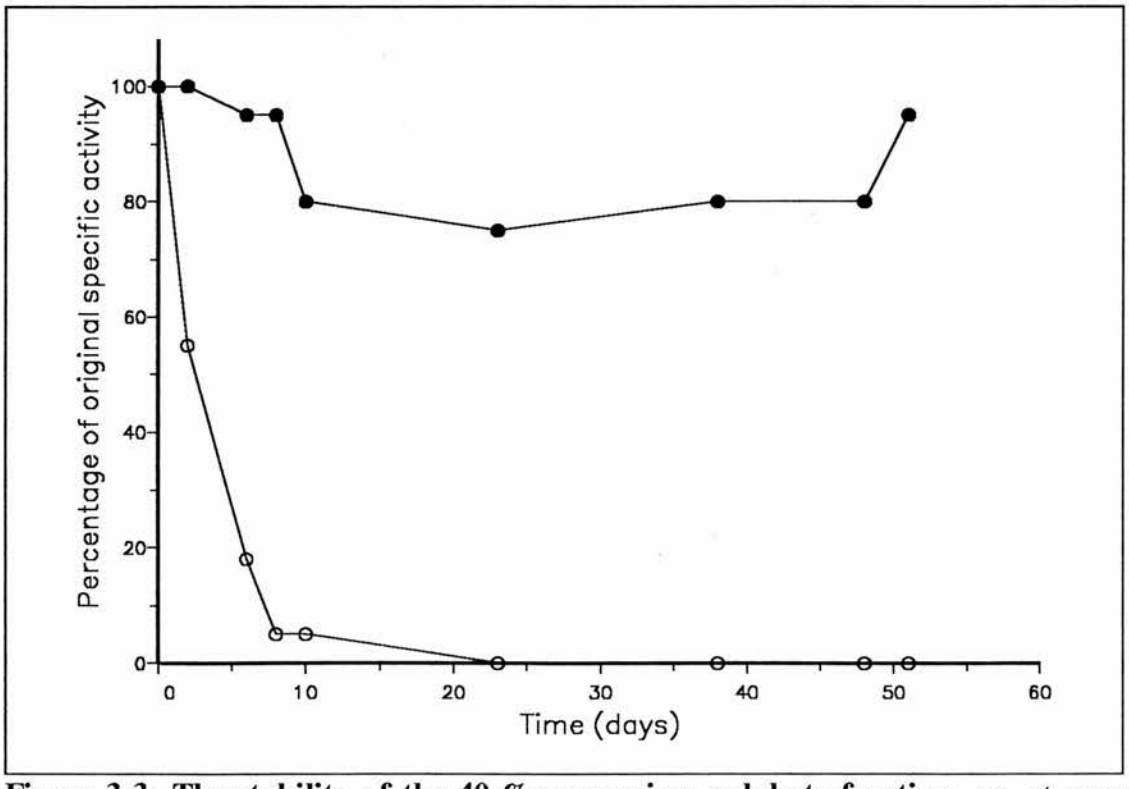


Figure 3.3 The stability of the 40 % ammonium sulphate fraction on storage at 4°C (○) and -16 °C (●). The specific activity at time zero was 1600 pmol/min/mg.

3.4 Cysteine sepharose affinity column

The aim of this purification procedure is to purify an ADP-ribosyltransferase which can modify a cysteine residue on a target protein. In this next step this property is exploited. The cysteine-sepharose may mimic the ADP-ribosyltransfer site of the target protein, thus allowing the enzyme to bind to the column. Non-specific NAD'ase activity and ADP-ribosyltransferases specific for arginine residues should not bind. Disulphide exchange may occur between protein disulphide bridges and the free cysteine exposed on the sepharose support. This interaction will be inhibited by the presence of free thiol groups in the buffers used.

3.4.1 Method

3.4.1.1 Preparation of Column

A cysteine Sepharose column was prepared by the reaction of free cysteine with cynanogen bromide-activated Sepharose 4B (Pharmacia), following the manufacturer's recommended protocol. Cynanogen bromide-activated Sepharose was swollen in 1 mM HCl and washed on a scintered glass filter with 1 mM HCl (200 mL/ 1g gel). The gel was washed with 0.1 M sodium carbonate pH 8.3, 0.5 M NaCl (coupling buffer) and then reacted with 2 mM cysteine in coupling buffer for 2 h at room temperature with gentle mixing. The recommended ligand concentration is 1 - 10 μmole ligand/ mL gel. Excess activated groups were blocked with 0.2 M glycine in coupling buffer for 16 h at 4 $^{\circ}$C with gentle shaking. The gel was washed with coupling buffer followed by 0.1 M acetate pH 4.0, 0.5 M NaCl for five cycles to remove blocking reagent.

20 mL of gel was poured into Pharmacia XL column (2.5 cm x 10 cm) and packed at 1 mL/min. The column was equilibrated with 50 mM potassium phosphate buffer pH7.0, 30% ethylene glycol. After use, the column was regenerated by successive washing with one column volume 1 M NaCl, one column volume water, one column volume 10 mM phosphate pH 7.0, 0.1 % 2-mercaptoethanol. Finally the gel was stored in 0.1 M acetate pH 4.0 at 4 $^{\circ}$C.

3.4.1.2 Measurement of Free Thiol

The availability of free thiol was tested using Ellman's reagent - 5,5'-dithiobis(2)nitrobenzoic acid) (DTNB). Disulphide exchange between DTNB and free thiol groups present in solution occurs, which results in the release of 5-thio-2-nitrobenzoic acid which is yellow coloured (ϵ_{412} = 13600 $M^{-1}cm^{-1}$). The method described by Habeeb was followed[130]. A stock solution containing 40 mg DTNB, dissolved in 30 mL 0.1 M sodium phosphate pH 7.2, was prepared. 0.1 mL of this reagent was added to 0.9 mL sample and the colour allowed to develop for 15 min, at room temperature. The absorbance at 412 nm was measured using a Cecil spectrophotometer. Micromolar concentrations of free thiol can be detected using this reagent.

3.4.1.3 Sample Treatment

The 40% ammonium sulphate pellet was solubilised and dialysed against buffer A to give a final protein concentration of 2-10 mg/mL. The material was applied slowly to the cysteine-Sepharose column (0.2 mL/min) to allow the protein to bind. The column was washed with ten column volumes of buffer A. Bound protein was eluted by successive washing with buffer A containing 0.1% 2-mercaptoethanol (buffer B), buffer B containing 0.1 M NaCl and buffer B containing 0.5 M NaCl.

3.4.2 Results

The cysteine sepharose affinity column was prepared following the manufacturer's protocol. The free thiol group is a reactive nucleophile and may react in preference to the amino group with the cyanogen bromide. Ellmans reagent was used to confirm the amount of free thiol bound to the column. 2.5 μmoles/ mL gel were detected. This is within the recommended concentration of affinity reagent 1 - 10 μmoles/ mL gel.

Protein binding to cysteine Sepharose is allowed to occur at pH 7.2. At this pH the carboxyl groups of cysteine and glycine (used to block the unreacted cyanogen bromide sites) will be negatively charged. To test whether the cysteine Sepharose column behaved as an affinity matrix rather than an ion exchange medium, the affinity

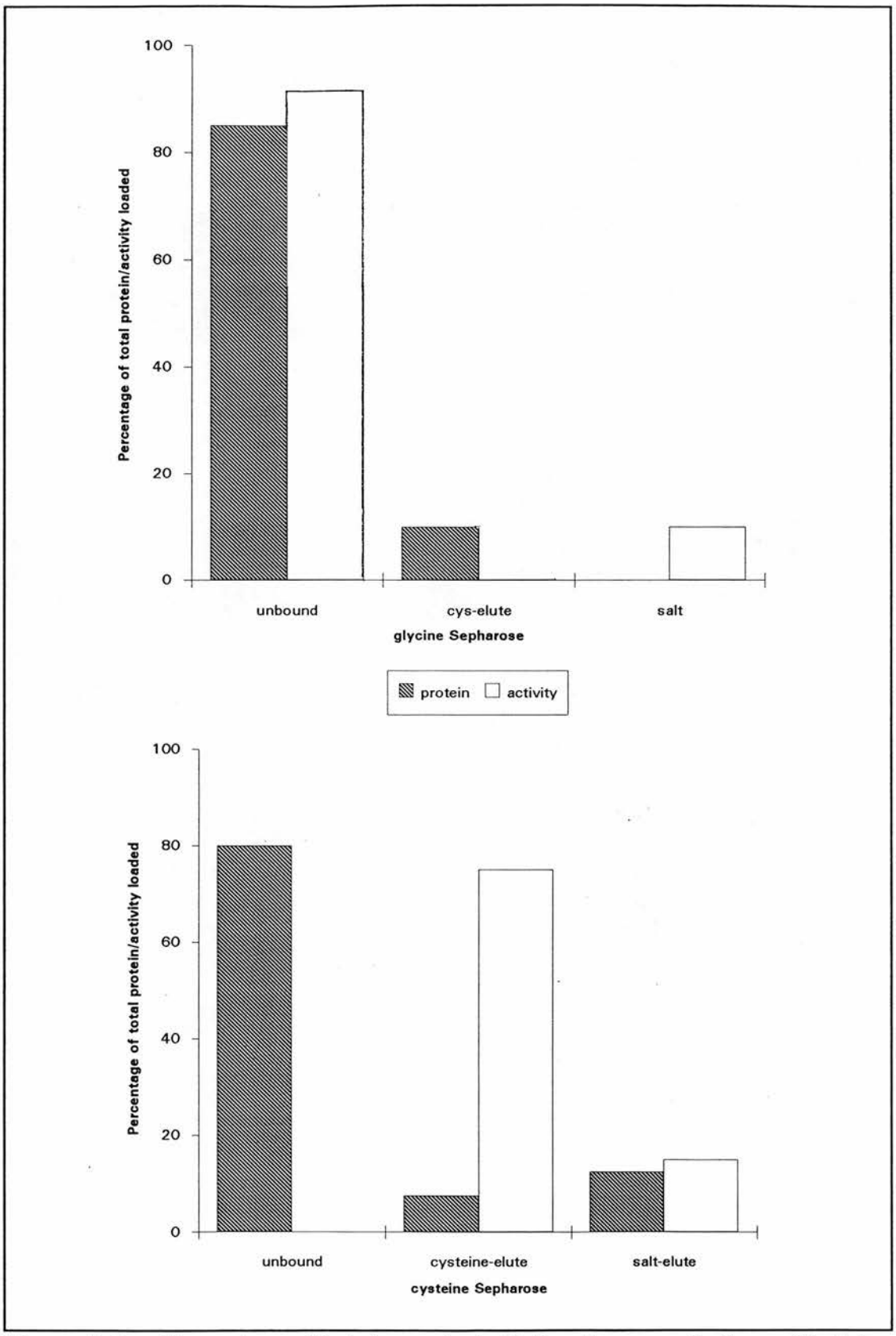


Figure 3.4 Percentage Protein and Activity Recovered from Cysteine Sepharose and Glycine Sepharose. 10 mg protein containing 10 nmol/min activity was loaded onto each column.

of the cysteine dependent activity for cysteine Sepharose and glycine Sepharose were compared. Trial columns were set up (2 mL each) and 10 mg protein, containing 10 nmol/min cysteine dependent NAD'ase activity was loaded. Figure 3.4 compares the percentage protein and activity recovered from the cysteine Sepharose column and the glycine blocked Sepharose column. The protein profiles are similar. 85% of the total protein did not bind to the column, 15% of the total protein bound to the column and was eluted with buffer B containing 0.5 M NaCl. Cysteine dependent activity, however bound to the cysteine sepharose column only and was eluted with buffer B containing 10 mM cysteine and 100 mM sodium chloride. Importantly haemoglobin and non-specific NAD'ase activity did not bind to either matrix.

Optimum binding of cysteine dependent NAD'ase to the cysteine sepharose column was achieved at protein concentrations 2-10 mg/mL and after allowing slow binding reactions to occur over one hour at zero flow rate. Low concentrations of protein lost activity rapidly and high concentrations of protein inhibited binding of cysteine dependent activity to the column, possibly by causing protein elution. Very dilute protein concentrations (less than 0.05 mg/mL) were obtained after this step, and the activity was extremely labile at 4 $^{\circ}$C. 50% of the cysteine dependent activity was lost in 3.5 h at 4 $^{\circ}$C in the presence of reducing reagent and is shown in Figure 3.5. Activity yields were variable; 10 - 40% for large scale preparations and 50 - 70% for small scale preparations. These observations can be primarily attributed to the labilility of the activity with time. Large prepartions take longer to apply to the column compared to small scale ones (10 h compared to 1 h). Some stabilisation of the enzyme was achieved by the introduction of ethylene glycol and protease inhibitors. Early experiments carried out in the absence of ethylene glycol lead to no recovery of activity. In the absence of protease inhibitors, low molecular weight bands running with the dye front were observed on SDS polyacrylamide gels, suggesting that the sample was susceptible to proteolysis, and this may be correlated with a reduction in activity yield. SDS polyacrylamide electrophoresis of this semi-purified fraction shows that three protein bands, 90 kDa, 45 kDa and 28 kDa are visible by silver staining (see Figure 3.8).

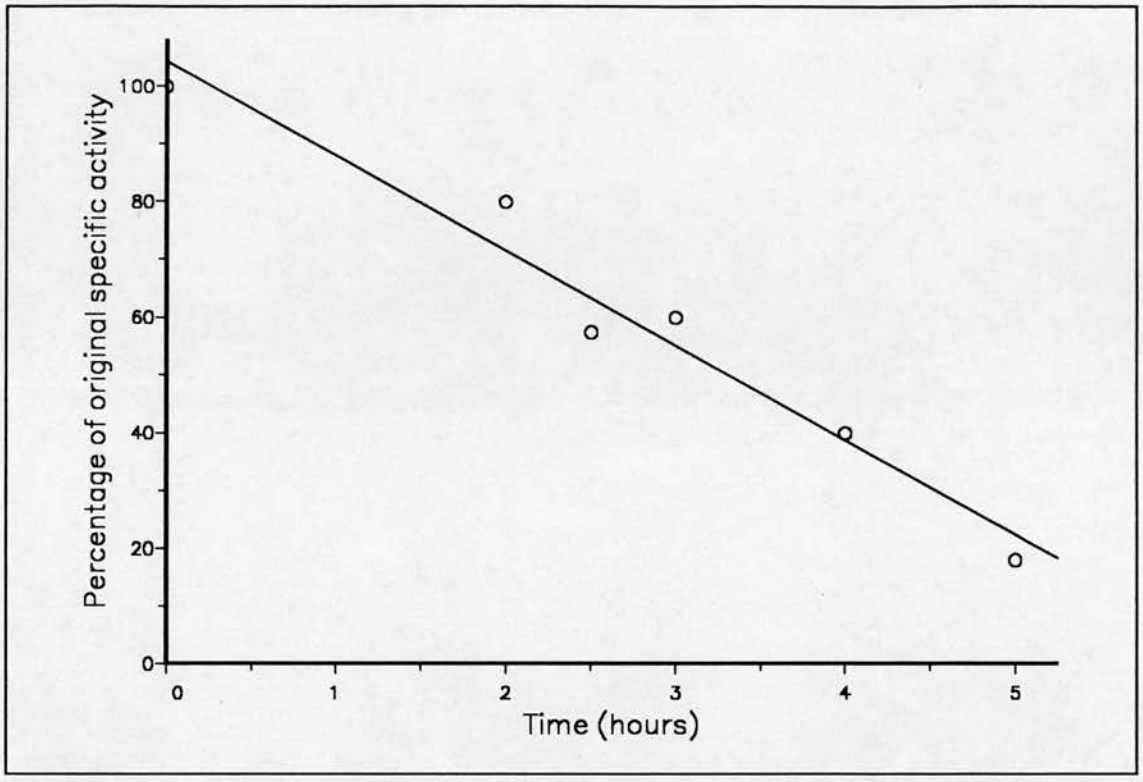


Figure 3.5 Time Dependent Inactivation of Semi-purified Fraction from Cysteine Sepharose.
The sample was stored in 50 mM potassium phosphate buffer, 10 mM cysteine, 100 mM NaCl, pH 7.2 at 4 °C

The cysteine dependent NAD'ase has been purified 20,000 fold with a specific activity 28.5 nmol/min/mg. It has been successfully separated from the non-specific NAD'ase activities which do not bind to the column and the major contaminating protein, haemoglobin, has been completely removed.

3.5 NAD^+ Binding Affinity Chromatography

Dye ligands have often been used to mimic NAD(H) binding and have successfully used to purify NAD(H) utilising enzymes. The affinity of the cysteine dependent NAD'ase for a variety of dyes was tested using the dyematrex (Amicon) ligand trial binding kit. The kit contains, 1 mL each of blueA, blueB, green, orange and red dyes covalently bound to Sepharose and also un-modified support as a control.

3.5.1 Method

The sample was dialysed against buffer A containing 0.1 % 2-mercaptoethanol to remove the salt. A 1 mL aliquot of known activity was applied to each column and binding allowed for 1 h. The columns were washed with 10 column volumes of buffer A to remove unbound material. Salt was added in a stepwise gradient, 0.1 M, 0.2 M, 0.5 M and 1M NaCl. Each fraction was tested for cysteine dependent NAD'ase as descibed in section 2.3.

3.5.2 Results

Table 3.2 Dye Affinity Ligand Binding Trial

LIGAND	% ACTIVITY UNBOUND	% ACTIVITY RECOVERED IN SALT WASHES
Blue A	50	30
Blue B	0	50
Green	0	25
Orange	0	70
Red	0	60
control	100	0

The cysteine dependent NAD'ase had a high affinity for all of the dye ligands tested, but the best recovery of activity was from Matrex orange. No activity was bound to the unmodified sepharose column, which shows that the cysteine dependent activity has an affinity for the dye ligands rather than non-specific binding to the Sepharose support. An added advantage of Matrex orange compared to Matrex red or blue B was noted when samples containing both cysteine dependent and independent NAD'ase activities were loaded onto these trial columns (results not shown). Cysteine dependent and independent NAD'ase activities had similar affinities

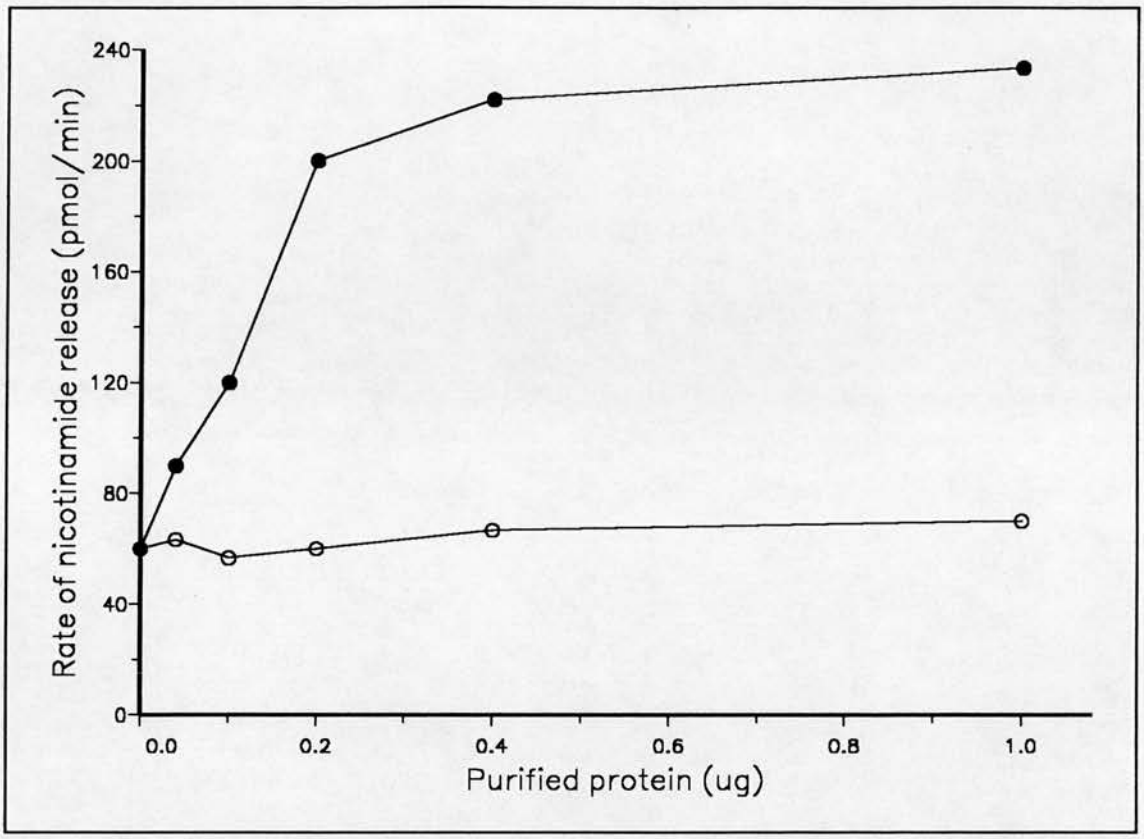


Figure 3.6 Dose Response Curve of Cysteine Dependent NAD'ase after purification on dye affinity column. Duplicate assays with (●) and without (○) 100 mM cysteine were performed.

for Matrex red and Matrex Blue, whereas the cysteine independent NAD'ase activity did not bind as tightly as the cysteine dependent activity to Matrex orange. Matrex orange gave the best recovery and best resolution of cysteine dependent activity of the ligands tested, and therefore was chosen for the final purification step.

To achieve a high recovery of activity for this step the time required for binding and elution from the column must be minimised because of the lability of the dilute semi-purified material obtained from the cysteine Sepharose column (Figure 3.5). This was achieved best by adding 2 mL Matrex orange gel with 30 mL semi-purified sample and rotating the mixture gently for 1 h to allow binding to occur. Then the gel was allowed to settle and the supernatant, containing unbound protein was removed. The gel was then washed and the bound activity eluted as described in section 3.5.1. Binding of the activity was improved by the presence of 0.01% 2-mercaptoethanol.

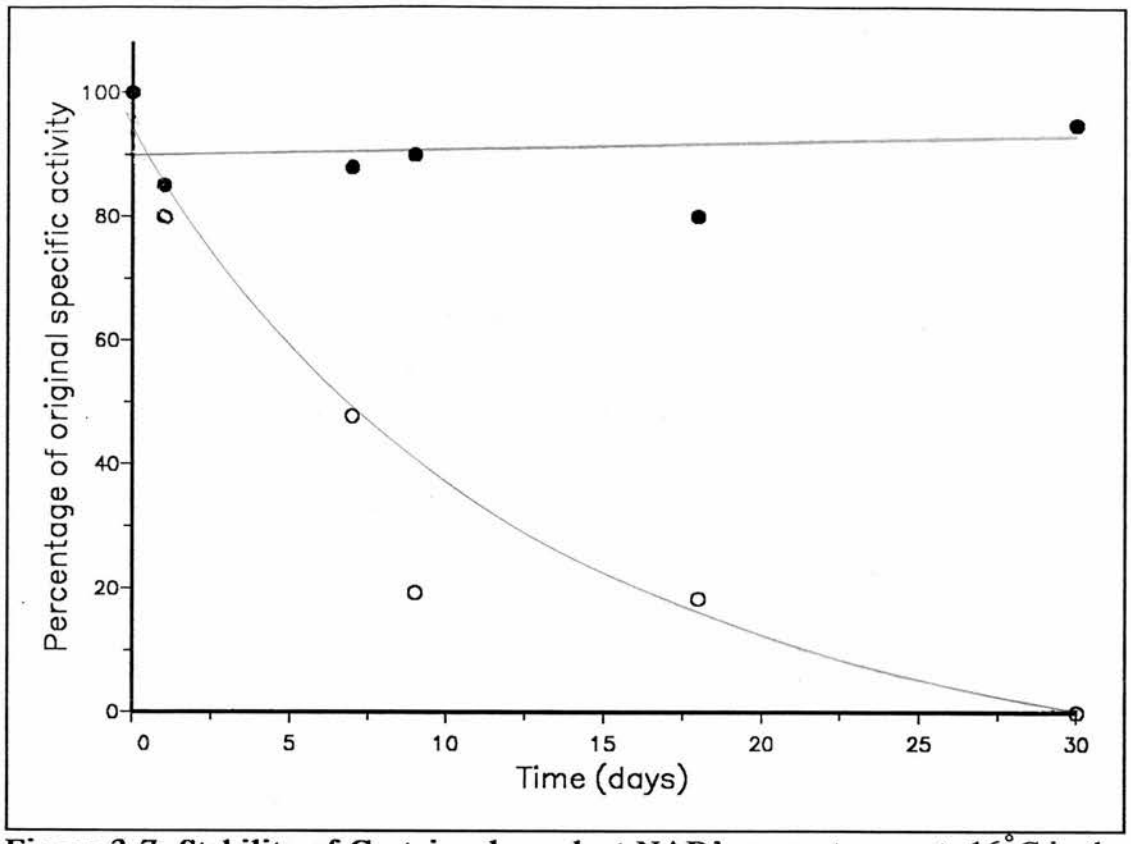


Figure 3.7 Stability of Cysteine dependent NAD'ase on storage at -16°C in the presence (○) or absence (●) of 0.01 %(v/v) 2-mercaptoethanol.

Cysteine dependent NAD'ase was eluted in buffer A containing 0.5 M NaCl, and 60-80 % recovery of activity was routinely achieved. Very low concentrations of protein were estimated by measuring the absorbance at 280 nm. 20-50 μg of protein were recovered and silver stained SDS polyacrylamide gels show a single band of 60 kDa. 70,000 fold purification has been achieved and the final product has a specific activity of 700 nmol/min/mg (Figure 3.6). No NAD'ase activity was observed in the absence of reducing reagent.

The sample was stored at -16°C in 50 mM potassium phosphate, 30 % ethyleneglycol, 0.5 M NaCl, 0.1 mM PMSF pH 7.0 in the absence of thiol. Figure 3.7 shows the loss of activity with time under these storage conditions. The presence of thiol reagent rapidly inactivates the enzyme, whereas in its absence, the enzyme can be stored for up to three months without loss of activity.

3.6 Summary of Purification Procedure

Summarised here is the procedure developed to purify a cysteine dependent NAD'ase activity from bovine erythrocytes. The typical protein recoveries and yields of activity are shown in table 3.3. Each step was carried out at 4 °C, unless otherwise stated, and 30 % (v/v) ethylene glycol and 0.1 mM PMSF were added to the buffers to help stabilise the enzyme activity.

Step one: Bovine erythrocytes lysate was prepared from 1 L of bovine blood, and protein was precipitated with 40% saturated solution of ammonium sulphate. The pellet was divided into six fractions and stored at -16°C. One aliquot was removed at a time and processed through the remaining purification steps.

Step two: The precipitated protein was solubilised in buffer A and dialysed to remove salts and traces of 2-mercaptoethanol. It was then applied to the cysteine-Sepharose column (bed volume 30 mL) at a flow rate of 0.5 mL/min.

Step Three: The dilute semi-purified protein activity was mixed with Matrex orange gel and allowed to bind. The bound activity was eluted with buffer A containing 0.5 M NaCl and was stored directly in this high salt buffer at -16°C.

Table 3.3 Purification Table

STEP	TOTAL PROTEIN (mg)	TOTAL ACTIVITY nmole/min	SPECIFIC ACTIVITY nmole/min/mg	FOLD PURIFICATION	YIELD %
LYSATE	16250	690	0.04	1	100
40% $AMMSO_4$	120	400	3	75	58
CYSTEINE-SEPHAROSE	0.6	152	250	6,250	22
MATREX ORANGE	0.06	116	1,900	47,500	16

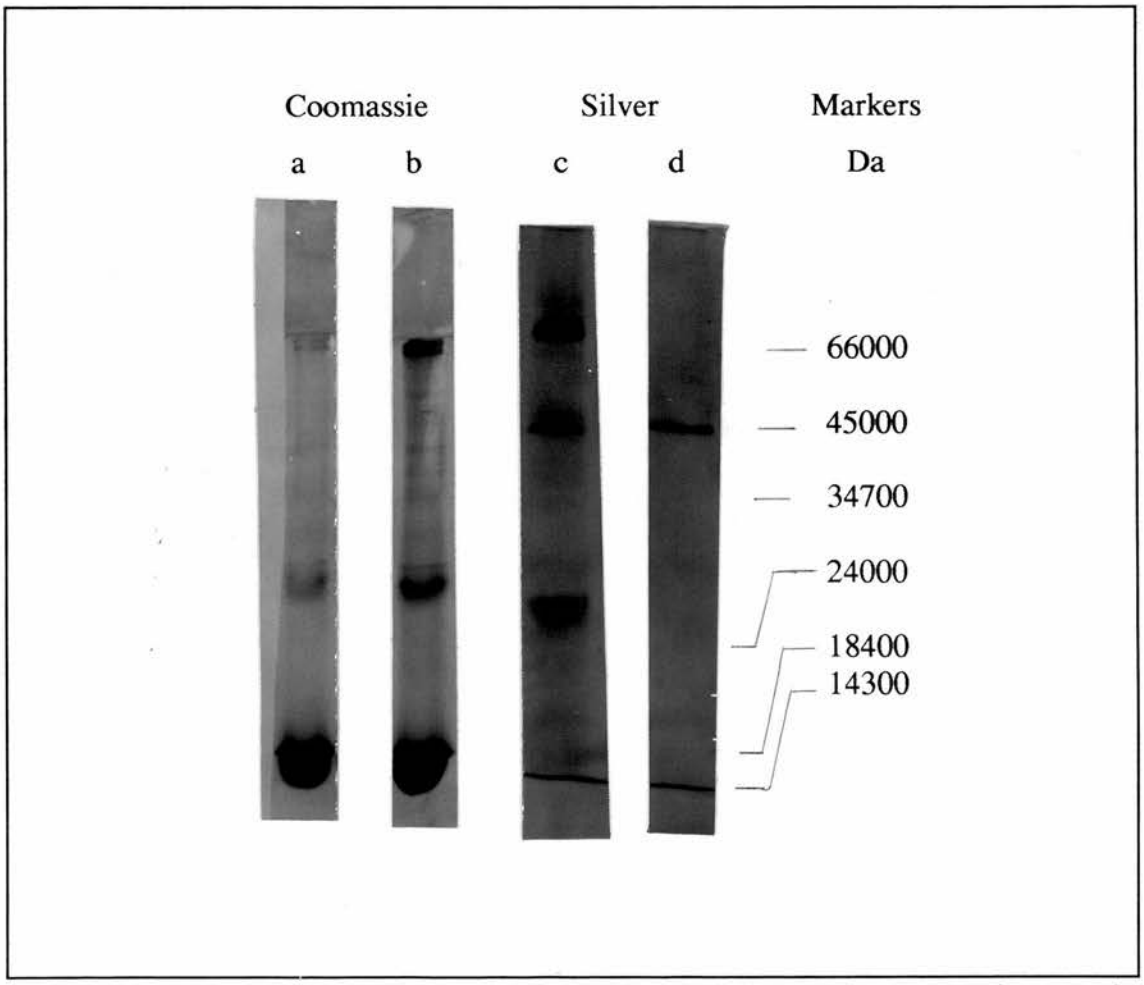


Figure 3.8 12 % SDS-PAGE gel of Purification Steps. Lanes (a) lysate, (b) ammonium sulphate, (c) cysteine sepharose and (d) matrex orange.

3.7 Preparation of Antibodies

3.7.1 Introduction

The production of antibodies against the purified cysteine dependent NAD'ase from bovine erythrocyte will provide a useful tool with which to study the distribution of this enzyme in other tissues. Polyclonal antibodies, raised against pure antigen will recognise a variety of epitopes. This should allow sensitive detection of cysteine dependent NAD'ase in crude mixtures, where cysteine dependent activity is too low to be detected. The antibodies were raised in mice because only a small quantity of purified material was available following the three step procedure described. The limited amount of hyperimmune sera produced would be enough to support the intended studies. The antigen used was inactive purified cysteine dependent NAD'ase which had suffered proteolysis to give 10-12 kDa fragments. Small, soluble, monomeric proteins generally induce a poor immune response because they are poorly phagocytosed. Therefore the initial immunisation was carried out in the presence of Freunds complete adjuvant. This contains heat killed *Mycobacterium tuberculosis*, which causes a non-specific stimulation of the immune response, and mineral oils which prevent rapid catabolism of the antigen. The polyclonal antibodies raised were then tested against antigen and active whole cysteine dependent NAD'ase on dot blots and Westerns.

3.7.2 Method

3.7.2.1 Raising the Antibody

150 μg cysteine dependent NAD'ase was concentrated to 1 mg/mL in 50 mM phosphate, pH 7.0, 30% ethylene glycol, 0.5 M NaCl. 30 μL aliquot was diluted five fold with phosphate buffered saline and then mixed with an equal volume of Freunds complete adjuvant. Pre-immune sera from three 8 week old BALB/C mice were collected by tail bleed. Each mouse was injected subcutaneously in scruff and flank with 5-10 μg antigen. An hyperimmune response was induced by injection of 5-10 μg antigen mixed with Freunds incomplete adjuvant into each mouse at 3 and 6 weeks. Test bleeds were taken at 6 weeks and the mice bled out at 8 weeks. Approximately 1.5 mL of blood was collected from each mouse. The blood was

allowed to clot for 30 min at 25°C. The clot was removed by centrifugation (10,000g, 10 min) and the serum was stored at -16°C.

3.7.2.2 Antibody Detection

1-5 μg of antigen were spotted onto nitrocellulose membrane and the excess binding sites were blocked by binding of 5 % (w/v) dried milk solids in Tris buffered saline (TBS) containing 0.1 % TWEEN (TBST). The pre-immmune and immune sera were diluted 1:200, 1:500, 1:1000 and 1:2000 with TBST and incubated with the dot blot for 2 hr. The dot blots were then treated as described in section 2.5.3 for treatment of western blots with second antibody and detection by ECL.

3.7.2.3 Immuno-precipitation of Cysteine Dependent NAD'ase Activity

Active cysteine dependent NAD'ase was incubated with 1:500 dilute immune and pre-immune mouse sera for 30 min, at 4 °C. Commercially available fixed protein A presenting S. aureus cells, Pansorbin (Calbiochem) were added. 10 μL (10% w/v) were added to 200 μL antigen, antibody mix and incubated for 30 min, at 4°C. The cells were removed by centrifugation (10,000g, 4 min) and the supernatants assayed for cysteine dependent activity.

3.7.3 Results

An hyper-immune response was induced in all of the mice. The antibodies raised were able to recognise the antigen and active cysteine dependent NAD'ase preparations on dot blots. No reaction was observed with the pre-immune sera. Figure 3.9 shows the reaction of the pooled immune sera and pooled pre-immune sera with native and denatured cysteine dependent NAD'ase as indicated. Unfortuneately no reaction with Western blotted proteins was achieved under comparable protein loading and antibody incubation conditions. SDS denatured antigen did not react with the antibody on dot blots, suggesting that the reactive epitopes were sensitive to denaturation.

Table 3.4 shows that 35% of the cysteine dependent NAD'ase activity remained after incubation with immune sera and protein A. 95 % remained in the presence of pre-immune sera and protein A. The activities observed were compared

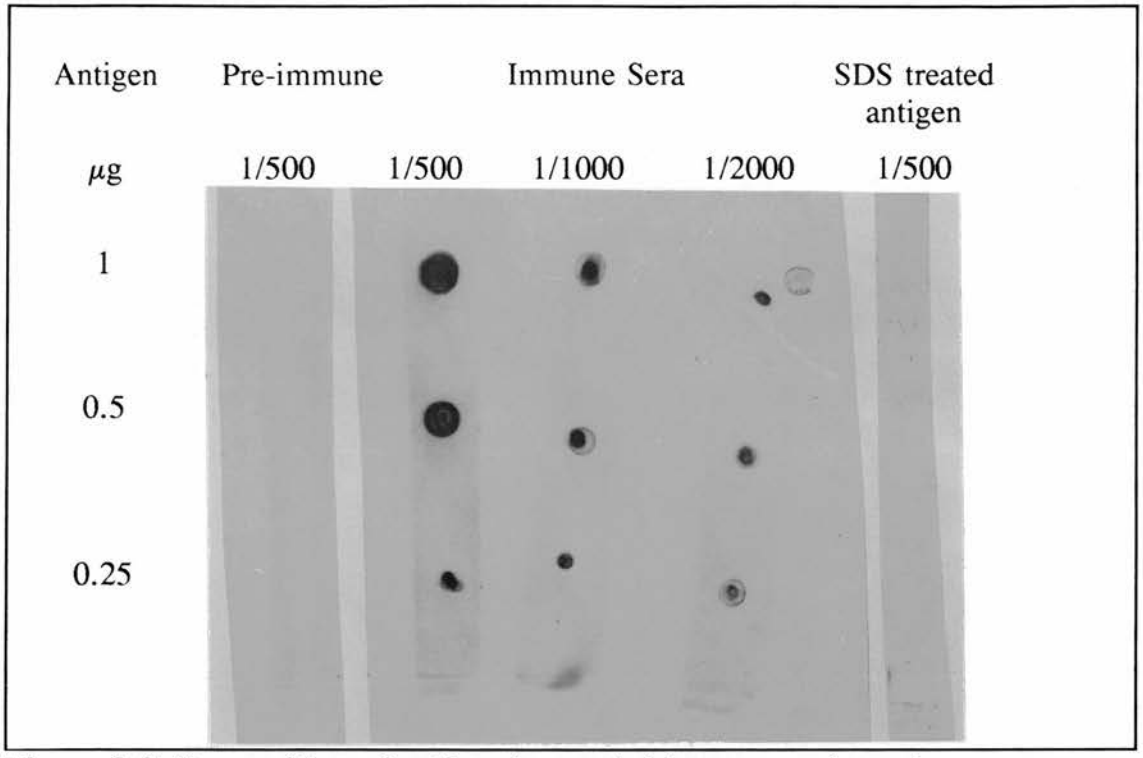


Figure 3.9 Recognition of antigen by pooled immune and pre-immune sera on dot blots

to cysteine dependent NAD'ase treated similarly, but in the absence of sera or protein A. This experiment confirmed that the antibodies raised against the degraded cysteine dependent NAD'ase did recognise whole, active cysteine dependent NAD'ase.

Table 3.4 Immuno-precipitation of Cysteine dependent NAD'ase activity

TREATMENT	PERCENTAGE OF THE ACTIVITY REMAINING IN SOLUTION
ENZYME UNTREATED	100
ENZYME CONTROL	100
ENZYME + PRE-IMMUNE SERA + PROTEIN A	95
ENZYME + IMMUNE SERA + PROTEIN A	35

3.8 Discussion

3.8.1 Bovine Erythrocyte Lysate as a Source for Cysteine Dependent NAD'ase

Examination of the lysate material illustrates two problems which have to be overcome in order to purify the cysteine dependent NAD'ase. First, haemoglobin is the single major contaminating protein. Coomassie staining of 10 μg lysate protein separated on 12% SDS polyacrylamide gel (Figure 3.8) shows a single band at 16 kDa. If efficient removal of haemoglobin could be obtained then 30,000 fold purification could be achieved in one step. However its presence in such excess, and at high concentration may cause it to behave anomously. Second, the cysteine dependent NAD'ase activity is not the only nicotinamide releasing enzyme present in bovine erythrocytes.

The NAD glycohydrolase assay measures the total nicotinamide released in the presence and absence of 100 mM cysteine, and the cysteine dependent activity is the difference between the two activities measured. Accurate determination of the cysteine dependent activity in crude fractions is difficult for three reasons. (1) The assay measures the release of nicotinamide and not the production of ADP-ribose-cysteine, directly. Several enzymes may be present in the lysate capable of catalysing the hydrolysis of NAD^+, for example, other ADP-ribosyltransferases such as the NAD^+: arginine ADP-ribosyltransferase[59] and NAD glycohdrolases which are particularly abundant in macrophage are also to be found in erythrocytes[131]. (2) The cysteine dependent release of nicotinamide must account for at least 15% of total nicotinamide released, in order that a significant difference between the [^{3}H] counts observed with and without cysteine in the assay may be detected. (3) It is possible that some NAD glycohydrolases may be inhibited by cysteine. Inhibition and activation of nicotinamide on addition of cysteine may cancel each other out such that no cysteine dependent activity is observed.

The specific activity of the total NAD'ase activity observed in the absence of cysteine is 60 pmol/min/mg protein. This is comparable to the activity quoted by Artman and Seeley[132] (40 pmol/min/mg) for NAD glycohydrolases in human erythrocytes (determined by the cyanide procedure[132]). This suggests that the erythrocyte preparation is free from platelet contamination, which have a ten fold

higher NAD'ase activity (450 pmol/min/mg)[132]. The specific activity of the cysteine dependent NAD'ase is 60 pmol/min/mg protein. This is four fold lower than that quoted by Tanuma *et al.*[75] (240 pmol/min/mg). This may reflect the different levels of expression of this enzyme activity in bovine and human. However Tanuma *et al.* used a different assay system. They claimed to have developed an assay system which measured the levels of ADP-ribose-cysteine directly, by the selective binding of product to DE52 ion exchange chromatography paper. Such an assay system was tried (results not shown), but no selectivity could be obtained; ADP-ribose bound as well as ADP-ribose-cysteine. If Tanuma's measurement is treated as total NAD'ase activity in the presence of cysteine, then the specific activities observed in human and bovine blood are similar. These data suggest that bovine erythrocytes are a suitable source for the purification of a cysteine dependent NAD'ase.

3.8.2 Ammonium Sulphate Precipitation

Ammonium sulphate precipitation was used as a crude fractionation step to concentrate the dilute cysteine dependent NAD'ase away from the main contaminating protein, haemoglobin. This step proved very successful on two counts. First, 75 fold purification was achieved, and 60-80% of the activity was recovered. Approximately 90% of the total haemoglobin and some of the non-specific NAD'ase activity were removed by this step. Second, fortuneately the 40 % ammonium sulphate pellet could be stored at -16°C for several months without loss of activity. This allowed bulk preparation of bovine erythrocyte lysate which could then be stored as manageable aliquots in the form of the ammonium sulphate precipitate and defrosted as required for further purification.

Previous protocols described by Moss *et al.*[59] and Tanuma *et al.*[75], for the purification of endogenous ADP-ribosyltransferases do not involve ammonium sulphate precipitation. The first step is binding to an hydrophobic support in batch. 60 % yields are quoted and similar fold purification (70 fold) achieved. A large volume of support is required for application of 1 L lysate which requires long process times for binding and elution of activity. This process results in very dilute protein on elution of the bound activity from the hydrophobic support. The time involved and the dilution factor made this step impracticable for the purification of

the cysteine dependent activity observed in bovine erythrocytes, which is extremely labile. The ammonium sulphate precipition provided a good alternative, by concentrating the activity and stabilising it.

3.8.3 Cysteine Sepharose Affinity Chromatography

Tight binding of cysteine dependent NAD'ase onto the cysteine Sepharose column, but no binding to the control support (glycine blocked Sepharose) was observed. Elution was achieved by the addition of reducing reagent (0.1 % 2-mercaptoethanol or 10 mM cysteine) and a high concentration of salt (0.5 M NaCl). Reducing reagent on its own was not sufficient to displace the bound activity. These data suggest that the strategy of using cysteine Sepharose to mimic the target protein has worked. Simple thiol exchange may also play a role, however, as it was observed that, binding of cysteine dependent NAD'ase to cysteine Sepharose was inhibited in the presence of reducing reagent. In this step the cysteine dependent NAD'ase is purified 6250 fold with a specific activity 250 nmol/min/mg. Three bands are visible with silver staining 90 kDa, 45 kDa and 28 kDa (Figure 3.8). It has been successfully separated from the major contaminating protein, haemoglobin and also, clearly separated from the non specific NAD'ase activities. No NAD'ase activity was observed for this semi-pure fraction in the absence of cysteine. Yields obtained for this step are variable 10-60%. Loss of activity could sometimes be correlated with evidence of proteolysis. Silver stained gels showed the appearance of a 24 kDa band and a low molecular weight fragment running with the dye front (not shown). The addition of protease inhibitors PMSF, EDTA and benzamidine greatly reduced this proteolysis. However activity was still extremely labile.

3.8.4 NAD^+ Affinity Chromatography

The final step involves binding of cysteine dependent activity to a dye ligand. The commercially available dyes have often been used for the purification of NAD(H) or nucleotide binding enzymes, eg malate dehydrogenase, citrate synthetase and adenylate kinase. Blue Sepharose was used successfully to purify the NAD^+: cysteine ADP-ribosyltransferase from human erythrocytes[76, 77], however an orange dye (Matrex orange) was found to have better binding characteristics for the bovine cysteine

dependent NAD'ase. The small volume of gel matrix used acts as a concentrating step, reducing the 30 mL of dilute activity, from the cysteine sepharose column, to 1.5 mL. Good recovery of activity applied to the column was routinely obtained (70 - 90%). The protein concentration was estimated from the absorbance at 280 nm. Separation of the purified fraction on 12% SDS polyacrylamide gel showed a single band with silver staining. A molecular mass of 45 kDa was estimated by comparison with the relative motilities of known molecular weight standards. This is higher than may have been expected. Previously purified endogenous ADP-ribosyltransferases had reported molecular weights of 28 kDa, similar to that of the S1 and A protomer catalytically active subunits of pertussis toxin and cholera toxin respectively. This suggests that a novel cysteine dependent NAD'ase has been purified.

3.8.5 The Three Step Purification Procedure

The three step purification procedure described has successfully isolated a cysteine dependent NAD'ase from bovine erythrocytes. The fold purifcation (47,500) and recovered protein (20-50 μg) are comparable to those quoted by Tanuma *et al.*[76, 77] for his prolonged five step purification of a NAD: cysteine ADP-ribosyltransferase from human erythrocytes (fold purification 35,000; protein 20-30 μg). After two steps, the major contaminating protein, haemoglobin was completely removed, and also the non-specific NAD'ase and cysteine dependent NAD'ase activity were resolved. The most important factor leading to the success of this purification procedure came from stabilising the enzyme activity. The addition of ethylene glycol and protease inhibitors and the finding of storage conditions which minimised loss of activity were contributing factors. No enzyme activity was recovered in the absence of ethylene glycol. The protein recovered from cysteine sepharose was very dilute (0.02-0.05 mg/mL), and in this form it was highly susceptible to proteolysis. The addition of protease inhibitors was essential to prevent this. The purified enzyme could be stored without loss of activity for up to three months in the presence of 30% ethylene glycol, 0.5 M NaCl. The presence of 10 mM cysteine or 0.01% 2-mercaptoethanol caused the loss of enzyme activity. The activity was also less stable in the absence of high salt concentrations. Time is another important factor. 50% loss of activity in 3.5 h on storage at 4 $^{\circ}$C was observed. Minimising the time taken to do

the preparation and storing the ammonium sulphate pellet at -16°C until required greatly improved activity yields.

3.8.6 Polyclonal Antibodies raised in mice

A disappointing result was obtained for the reactivity of the polyclonal antibodies raised in mouse with the antigen on Western blot analysis. Antibody recognition was apparently blocked by denaturation. Dot blot analysis of native and denatured cysteine dependent NAD'ase showed strong antibody recognition with the native protein only. The small peptide products used were probably not ideal immunigens. A good immunogen has three propeties. One, its epitope must be recognised by cell surface antibody found on B cells. Two, it must have a site which may be recognised simultaneously by Class II and T cell receptors. Three, it must be degradable. The chances of a good immune response are improved by optimizing phagocytosis conditions. In this protocol Freunds complete adjuvant was used to trap the antigen and stimulate host immune response. Alternatively, the antigen could have been modified by linking it to a carrier or using nitrocellulose bound material[133,134]. Raising antibody against denatured protein bound to nitrocellulose may give a positive result on subsequent Western blot analysis.

COMPARISON OF NAD GLYCOHYDROLASE ACTIVITIES OF PERTUSSIS TOXIN AND CYSTEINE DEPENDENT NAD'ASE PURIFIED FROM BOVINE ERYTHROCYTES

4.1 Introduction

NAD glycohydrolase activity describes the hydrolysis of NAD^+ by cleavage of the N glycosidic linkage between ADP-ribose and nicotinamide (1). Enzymes catalysing this reaction are widely distributed[135]. NAD glycohydrolases (E.C. 3.2.2.5.) are found in all mammalian cells and are particularly abundant in macrophage. ADP-ribosyltransferases[136], (E.C. 2.4.2.30, E.C. 2.4.2.31) catalyse the transfer of ADP-ribose from NAD^+ to an acceptor molecule with concomittant release of nicotinamide and H^+ (2), and also exhibit NAD glycohydrolase activity to some degree in the absence of acceptor.

$$NAD^+ + H_2O \rightleftharpoons \text{ADP-ribose} + \text{nicotinamide} + H^+ \qquad (1)$$

$$NAD^+ + \text{Acceptor} \rightleftharpoons \text{ADP-ribose-Acceptor} + \text{nicotinamide} + H^+ \qquad (2)$$

NAD glycohydrolase activity can be measured easily by separation on an ion exchange resin of [^{3}H]nicotinamide released from [^{3}H]NAD^+. Although this assay does not distinguish between the above reactions it has been extensively used for kinetic studies of both bacterial protein toxins and endogenous ADP-ribosyl-transferases. The reaction is generally regarded as a model to analyse the initial events in the enzymatic mechanism of ADP-ribosylation.

The cloning of the pertussis toxin operon was achieved independently by two groups[10,11]. Site directed mutagenesis and the study of deletion mutants has advanced the knowledge of structure function relationships of S1 greatly, but the catalytic mechanism is still poorly understood (this is discussed in detail in section 1.2.3). Important residues have been identified which are essential for NAD'ase activity in pertussis toxin. These are Arg-9, Trp-26, His-35, and Glu-129[137]. The first three

form part of two regions of eight amino acids long which are homologous to cholera toxin A protomer which has been postulated to form part of a common N-terminal NAD^+ binding site.

Glu-129 has been shown to be involved in catalysis. Photoaffinity labelling of pertussis toxin resulted in covalent binding of the labelled nicotinamide moiety of NAD^+ to Glu-129[138]. Deletion or replacement of Glu-129 with apartic acid resulted in a drastically reduced enzymatic activity but had little effect on the binding of NAD^+[139]. The role of glutamate in the catalytic mechanism is a matter of some debate. It may serve as a general base to deprotonate the ADP-ribosyl acceptor substrates or it may stabilize an oxocarbonium ion transition state intermediate of NAD^+ in the active site. A catalytically important glutamate residue is observed for other bacterial toxins namely Glu-148 and Glu-553 of diphtheria toxin[140] and pseudomonas exotoxin A[141] respectively. It seems that the amino-acid residues involved in the catalytic mechanism are conserved amongst the ADP-ribosylating toxins despite low degrees of significant similarity in the primary amino acid sequence. In the absence of any sequence data concerning the endogenous ADP-ribosyltransferases, it is interesting to speculate whether these same residues will be conserved for these mammalian enzymes.

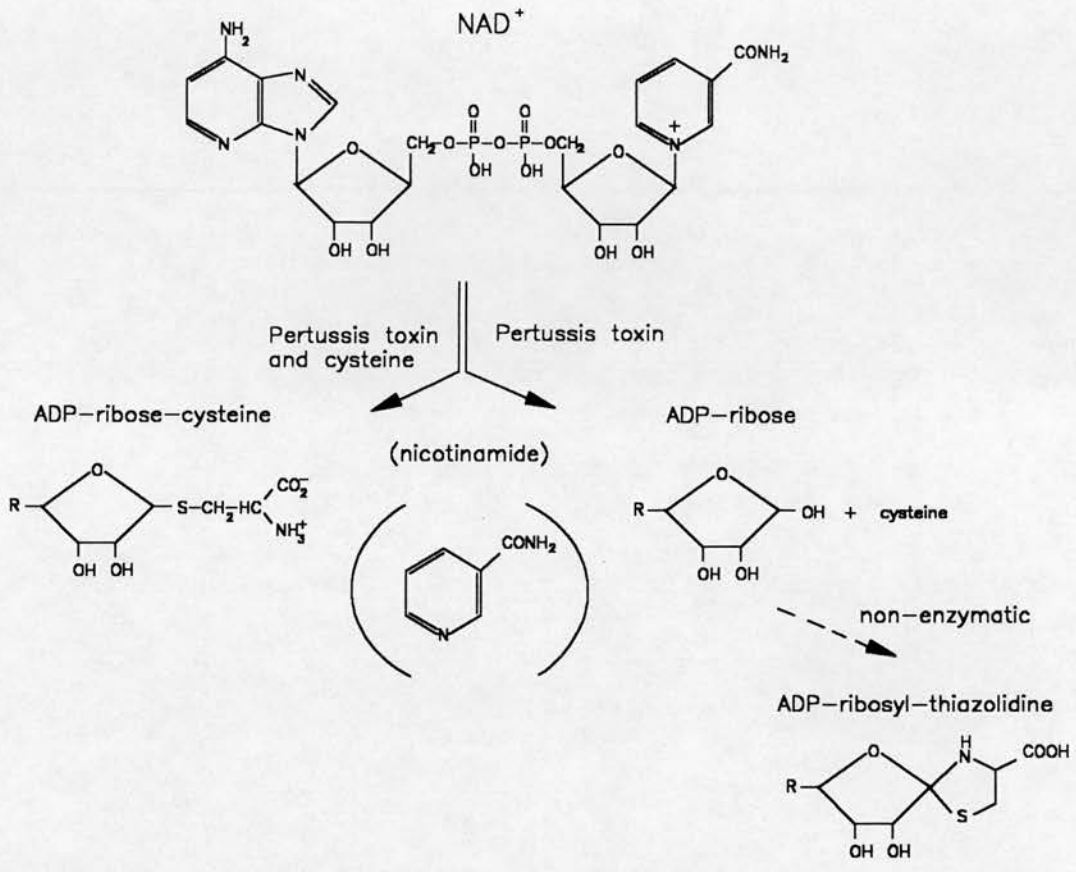
Pertussis toxin is inactive in the absence of reducing reagent. Examination of the crystal structure of pertussis toxin shows that reduction of the disulphide bridge between cysteine-41 and cysteine-200 opens up the NAD^+ binding site and allows the access of the substrate to the active site[16]. Activation procedures which involved incubating pertussis toxin with 100 mM DTT for 3 hours were originally described to activate the toxin[142]. These harsh conditions were required to reduce intra-subunit disulphide bonds, resulting in a conformational change which allowed the release of the S1 subunit from the B oligomer. Later, activation of the toxin was found to be promoted by ATP and detergent *eg.*CHAPS[143] by promoting the dissociation of the S1 subunit from the B oligomer[144] and stabilising the S1 subunit in solution. The addition of these agents allowed much lower concentrations of DTT (10 mM) and shorter incubation times (10 min) to be employed. Kaslow *et al.*[22] demonstrated that membrane lipids and cellular concentrations of glutathione and ATP would be sufficient to activate the ADP-ribosyltransferase activity of pertussis toxin in the cell.

However, following this activation procedure, maximal NAD glycohydrolase activity still required the presence of high concentrations of DTT (250 mM), far higher than would be expected for reduction of a disulphide bond.

The role of thiol compounds in the NAD'ase activity of pertussis toxin may be two fold. They may, not only, activate the enzyme by reduction of a disulphide bond in the S1 subunit, but also, they may act as an acceptor for ADP-ribose, thus behaving as a substrate. Lobban and van Heyningen[121], in this laboratory, showed that cysteine did indeed show substrate like behaviour, although it had a very low affinity (K_m = 100 mM), and were able to isolate a product on HPLC which contained both cysteine and ADP-ribose. Treatment of this ADP-ribosyl-cysteine compound with phosphodiesterase released 5'AMP, leading them to conclude that cysteine was an acceptor for ADP-ribose. As an extension to this work the ability of other small thiol containing compounds to behave as substrates and exhibit much higher affinity for pertussis toxin was investigated. This approach is analogous to the model assay system developed for cholera toxin[145], where arginine-methylester and other gaunidino containing compounds were found to be acceptors for ADP-ribose.

Another approach taken was to mimic the site of ADP-ribosylation of G_i α, the protein substrate of pertussis toxin. This C-terminal region is essential for ADP-ribosylation. The C-terminal cysteine is the site of ADP-ribosylation[9], and ADP-ribosylation of G_i α is sensitive to mutations in three out of the four C-terminal amino acids. Substitution of a glycine residue adjacent to the cysteine for apartic acid or deletion of the the last two terminal amino acids has been shown to block ADP-ribosylation of G_i α[146]. Synthetic fragments derived from this region may mimic functional aspects of this domain. A hexamer and dodecamer peptide of the C-terminal of G_i α were synthesised and tested for their ability to act as acceptors for ADP-ribose.

Since this work was undertaken, McDonald *et al.*[147] have reported that the ADP-ribosylcysteine product identified on HPLC may have been generated non-enzymatically. They proposed that the amino group of cysteine could react with the anomeric carbon of free ADP-ribose generated in the assay by the NAD glycohydrolase activity of pertussis toxin. The carbinolamine produced undergoes a series of re-arrangements via a Schiffs base to form ADP-ribosylthiazolidine product.



The Possible Enzymatic and Non-enzymatic, ADP-ribose and Cysteine Containing Products from the Incubation of Pertussis Toxin with NAD^+ and Cysteine.

The structure of this product was confirmed by ^1H NMR.

Following the investigation of thiol reagents as substrates for pertussis toxin a study of the kinetic properties of the cysteine dependent NAD'ase purified from bovine erythrocytes was made. This was carried out to confirm that a "pertussis toxin-like" activity rather than a "cholera toxin-like" activity had been purified, *ie* the enzyme would have a high affinity for NAD^+ and cysteine would be able to act as an acceptor for ADP-ribose, but not arginine or any other amino acid. The substrate affinity of NAD^+, cysteine and other compounds were found and compared to those of pertussis toxin and other known ADP-ribosyltransferases or NAD'ases.

4.2 Alternative Thiol Substrates for Pertussis Toxin

4.2.1 Method

Cysteine methylester, S-carboxymethylester, cys-gly dipeptide and glutathione (all purchased from Sigma), are derivatives of cysteine which were tested using the standard NAD'ase assay procedure described in section 2.3. Compounds with thiol-containing ring structures mercapto-benzamide, mercapto-pyridine and mercapto-benzamidine were also tested. All solutions were made fresh and de-gassed to prevent oxidising reactions. The C-terminal hexapeptide and dodecapeptide of $G_i\alpha$ were synthesised in collaboration with Professor Ramage's group (University of Edinburgh, Centre for Molecular Recognition, Dept. Chemistry, Kings Buildings, Edinburgh). Only 10 mg of each peptide was synthesised using an automated peptide synthesiser (Applied Biosystems). A maximum of 1 mM was added to the NAD'ase assay. Direct modification of the peptide by ADP-ribose was identified by HPLC.

4.2.1.1 ADP-ribosylation of Peptide: Incubation

1 mg dodecamer or hexamer were incubated in a total reaction volume of 300 μL containing 50 mM potassium phosphate buffer, pH 7.2, 100 μM [Adenine-2,8-^{3}H]NAD$^+$ (0.1 Ci/mmole, 30,000 cpm), 1.5 mg BSA, 0.3% CHAPS, 30 μM ATP and 20 mM DTT for 18 hours at 30°C. Each reaction was repeated in duplicate in the presence and absence of 10 μg pertussis toxin. The reaction conditions described would allow as little as 0.02% of the modified peptide to be detected from the counts associated after separation of the products on a HPLC reverse phase column.

4.2.1.2 Identification ADP-ribosyl-Product using HPLC

After the incubation pertussis toxin and BSA were removed from the reaction mix by filtration of the products through a membrane with a cut-off *Mr* 10,000 (Amicon microconcentrator). The products of the NAD glycohydrolase assay were separated out on an ODS-HYP 2734 (Highchrom) reverse-phase column using Altex equipment as described in section 2.4. Ideally, the relative retention times of the charged nucleotides on the reverse-phase support are maximised by the use of a

quarternary ammonium "solvophobic ion". For the purposes of this experiment, potassium phosphate buffer was used instead of ammonium phoshate. Ammonium phosphate was found to react with the *ortho*-phthaldialdehyde (OPA) reagent used to detect the peptide. The change of buffer did not effect the elution times of NAD^+ and ADP-ribose standards (11.2 min and 5.2 min respectively). The optimum running conditions, for separation of the products was 1 mL/min, 1000 psi. NAD and ADP-ribose were eluted isocratically and detected at 245 nm. A 10 - 100% methanol gradient was then applied to elute the peptide.

4.2.1.3 Detection of Peptide

Both the peptide and the nucleotides present had high relative absorbances at the wave length of detection, 245 nm (λ_{max} 220 nm and 260 nm respectively). The presence of peptide was identified by modification of the α amino group using OPA[148]. This reagent reacts with α amino group of the peptide in the presence of 2-mercaptoethanol at pH 10 to form a highly fluorescent isoindole compound ($\lambda_{excitation}$ 360 nm and $\lambda_{emission}$ 455 nm) which may be detected using a fluorimeter (Perkin Elmer MA-3000). OPA was chosen in preference to other modifying reagents such as ninhydrin (less sensitive) or phenylisothiocyanate PITC (absorbs 254 nm). A 0.8 g/L stock solution of OPA was made in 0.1 M borate buffer, 0.02% 2-mercaptoethanol, pH 10. 0.2 mL fractions collected from HPLC were diluted to 3 mL with OPA and allowed to react at room temperature for 20 min. Detection of nanomolar quantities of peptide was possible.

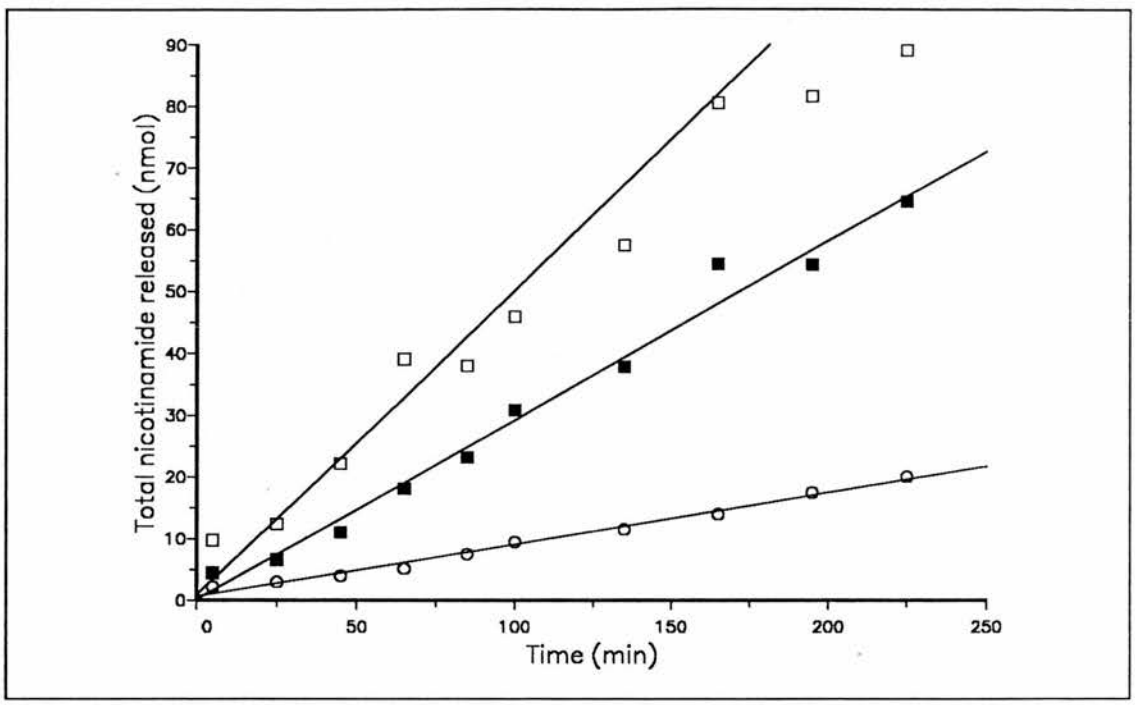


Figure 4.1 Time Course of Pertussis Toxin Catalysed NAD'ase Activity.
The nicotinamide released by the action of 20 μg pertussis toxin in the presence of 0 (○), 10 (■) and 100 mM (□) dithiothreitol was measured using the NAD'ase assay described in section 2.3.

4.2.2 Results

Before kinetic analysis of the potential substrates was investigated the time course of the pertussis toxin catalysed NAD'ase activity was determined. Conditions were found where the reaction was linear over the time course of the reaction and the maximum amount of hydrolysis of NAD^+ was limited to 20% of the total NAD^+ present in the assay. Figure 4.1 shows the release of nicotinamide with time by 20 μg of activated pertussis toxin (activated as described in section 2.2) in the presence of 100 mM DTT, 10 mM DTT and absence of DTT. The reaction was linear over a 180 min time period, beyond which the substrate became limiting. An incubation time of 90 min was chosen and was used routinely in subsequent experiments.

4.2.2.1 Apparent Substrate Kinetics

The rate of nicotinamide release catalysed by pertussis toxin in the presence of 0-500 mM concentrations of the potential substrates was tested. Cysteine, cysteinemethylester, cysteine-glycine dipeptide, glutathione and dithiothreitol all

showed substrate-like kinetics, ie the rate of nicotinamide released became saturating for increasing concentrations of compound added. The data were fitted to the Michaelis-Menten equation using non-linear least squares curve fitting (section 2.3.3). A linear transfomation of the data in the form of the Hanes plot ([S] versus [S]/V) are shown for each compound in Figure 4.2 through to Figure 4.6. The units for these plots were millimolar concentrations of thiol substrate on the x-axis, and $1x10^9$ min.L^{-1} substrate over rate on the y-axis. The data shown is a single data set representative of three separate experiments in which the parameters determined (K_m and V_{max}) varied within 10% of each other. The standard error shown for each parameter is a measure of the goodness of fit of the data for the Michaelis-Menten equation and not the error between experimentally determined values.

The apparent K_m and V_{max} values calculated are summarised in table 4.1. The cysteine-like structures showed similar apparent K_m values of 100 mM, however the presence of a free sulphydryl group was essential for apparent substrate kinetics to be observed; S-carboxymethyl-cysteine was inactive. The sulphur containing ring systems of mercapto-pyridine and mercapto-imidazole were also inactive; no increase

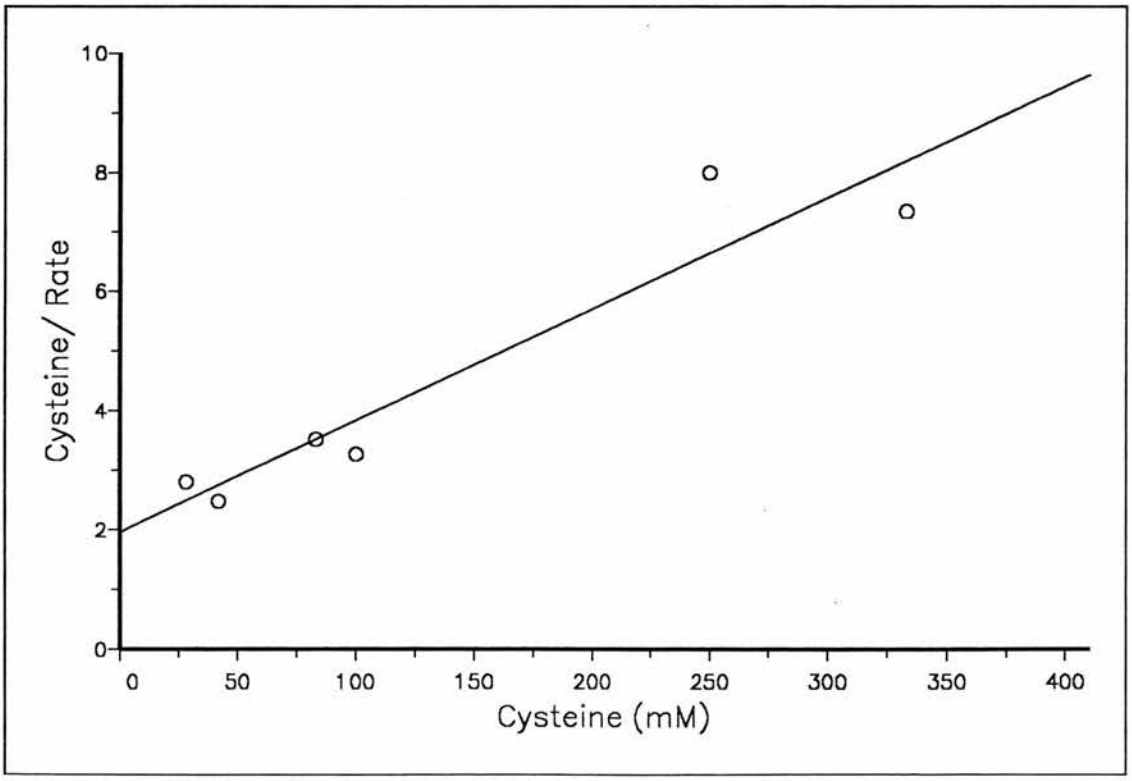


Figure 4.2 Hanes Plot for Cysteine from which a K_m of 109±30 mM, and V_{max} of 55±5 pmol min^{-1} was estimated.

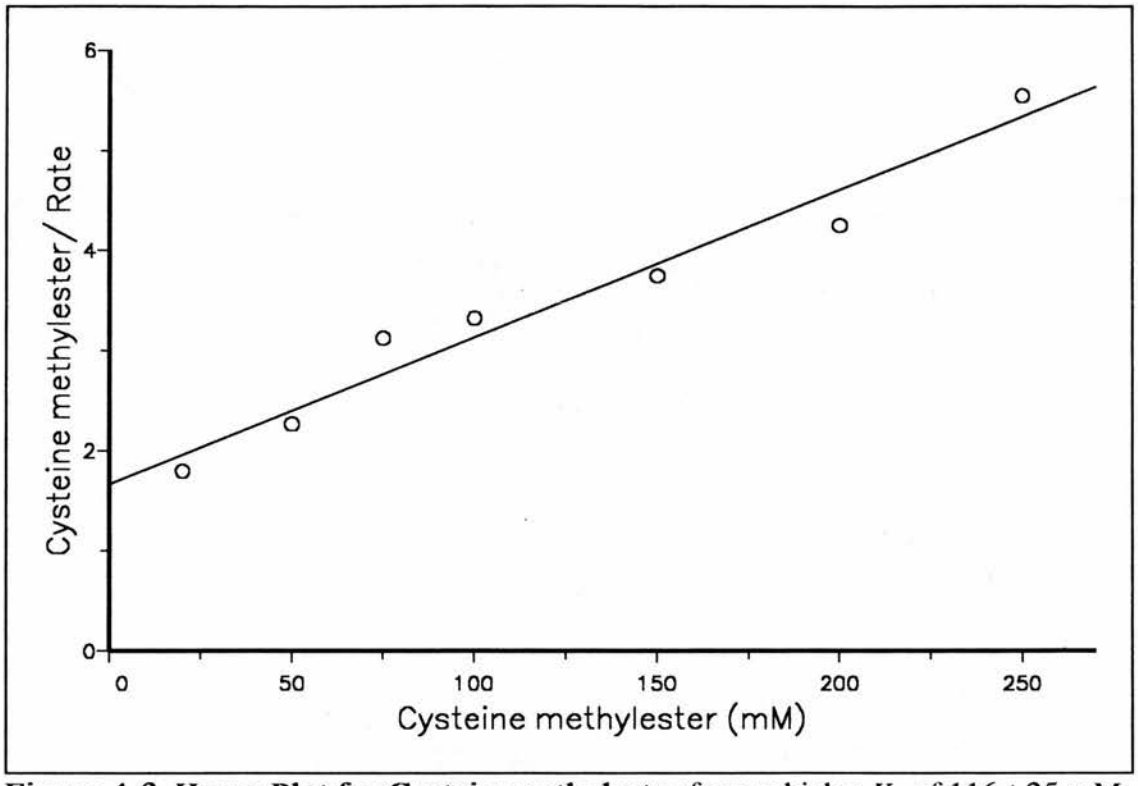


Figure 4.3 Hanes Plot for Cysteinemethylester from which a K_m of 116±25 mM, and V_{max} of 50±5 pmol min^{-1} was estimated.

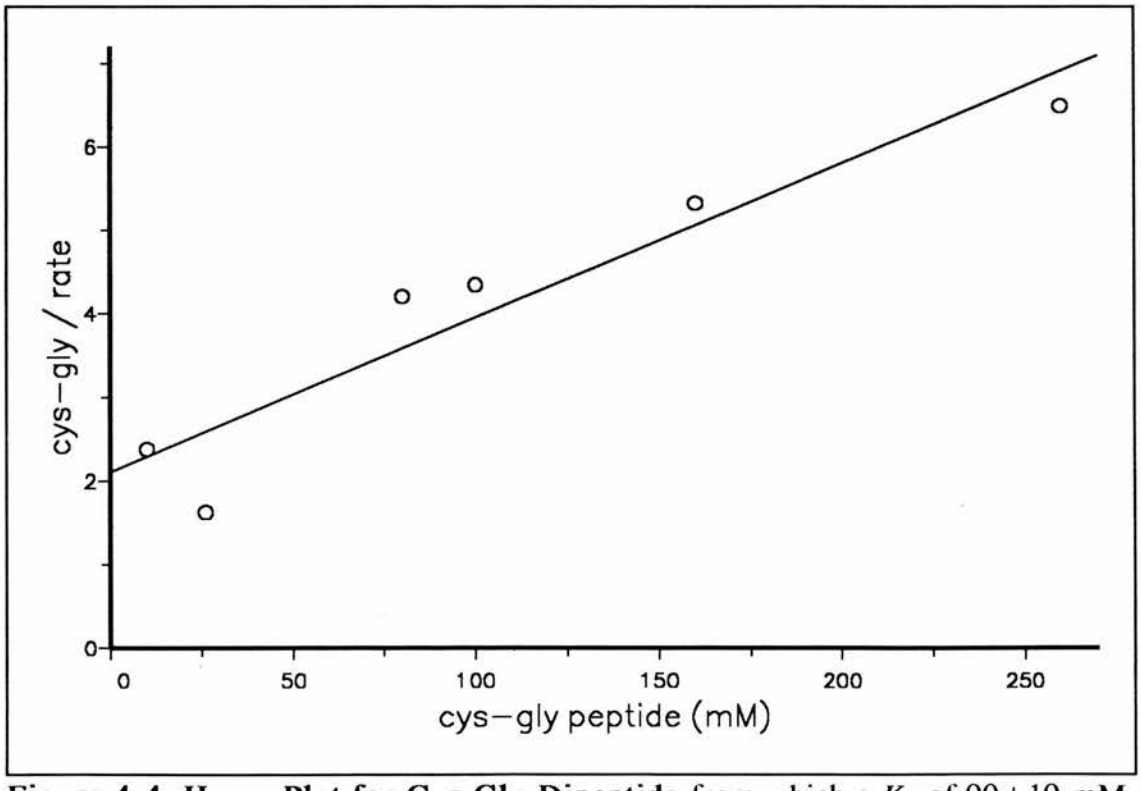


Figure 4.4 Hanes Plot for Cys-Gly Dipeptide from which a K_m of 90±19 mM, and V_{max} of 56±5 pmol min^{-1} was estimated.

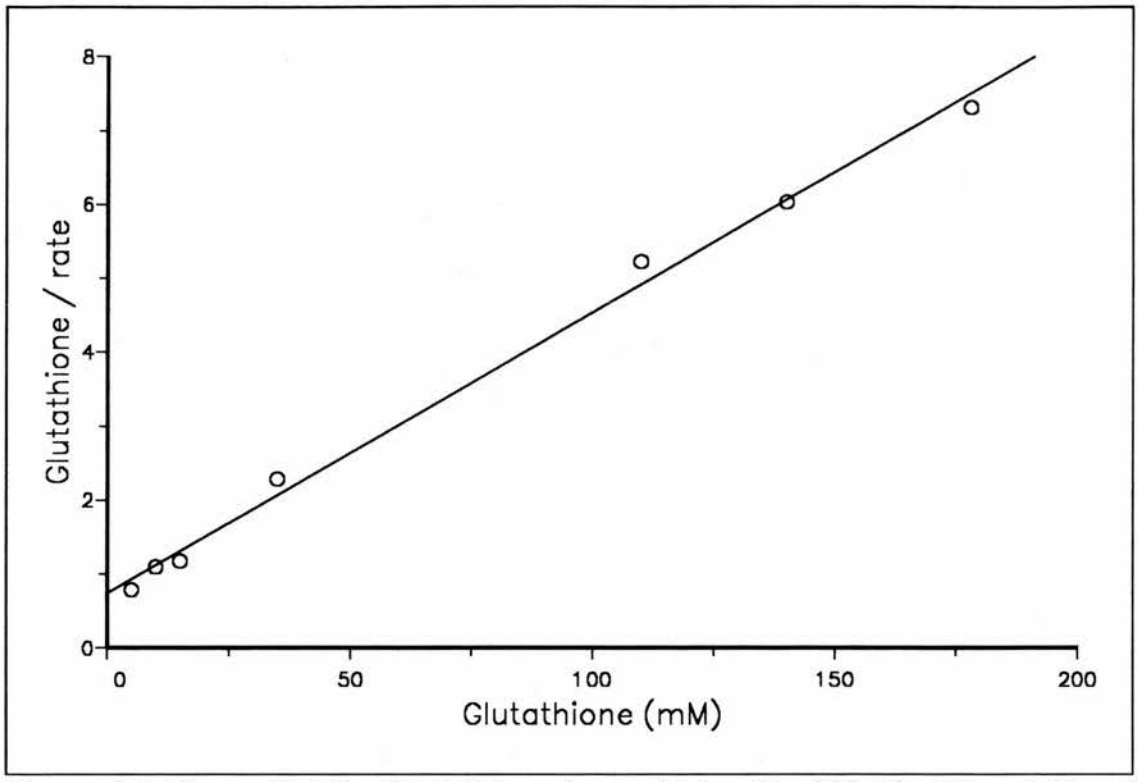


Figure 4.5 Hanes Plot for Glutathione from which a K_m of 20±3 mM, and V_{max} of 25±3 pmol min^{-1} was estimated.

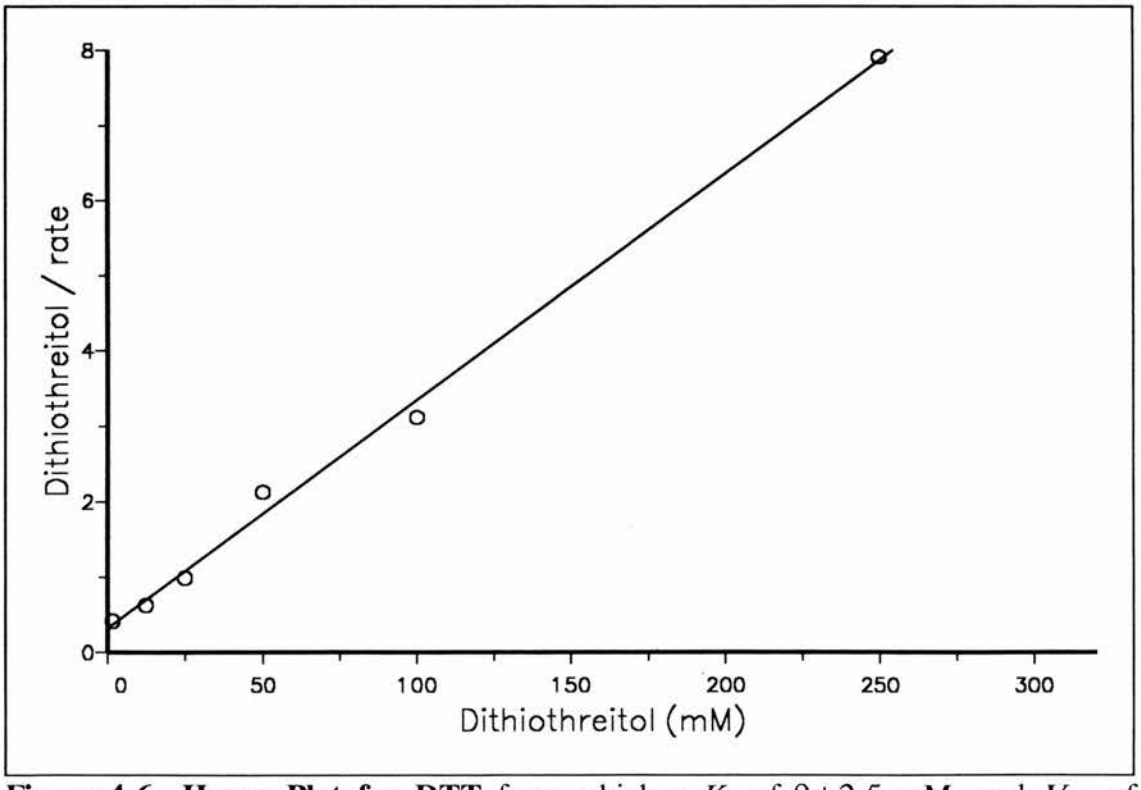


Figure 4.6 Hanes Plot for DTT from which a K_m of 9±2.5 mM, and V_{max} of 33±3 pmol min^{-1} was estimated.

in the rate of nicotinamide released was observed for additions of compound to a maximum of 500 mM.

4.2.2.2 Peptide Synthesis and Confirmation

The products of both syntheses ran as single peaks on C18-reverse phase HPLC column suggesting that a homogeneous product had been formed and no side chain branching reactions had occurred. The correct composition was confirmed by amino acid analysis and mass spectrophotometry. The relative proportions of each of the amino acids was confirmed by this method and no unspecified amino acids were present. The molecular mass of the product was compared to the theoretical expected for the required composition. Mass spectrometry of the dodecamer showed a single mass ion with a relative molecular mass of 1378.5 (calculated mass, 1377.69). Similarly the hexamer showed a single mass ion of 682 (calculated mass 681.82). These data were consistent with the peptides having the composition specified:

C-terminal dodecamer of $G_i\alpha$	I I K N N L K D C G L F
C-terminal hexamer of $G_i\alpha$	K D C G L F

4.2.2.3 Identification of ADP-Ribosyl Peptide by HPLC

The separation of NAD^+, ADP-ribose and ATP standards by isocratic elution from the reverse-phase HPLC column in 10 mM potassium di-hydrogen phosphate, 5 % methanol, pH 3.5 is shown in Figure 4.7. NAD^+ and ADP-ribose are well separated, and have elution times of 11.2 and 5.2 min respectively. Nicotinamide, the product of hydrolysis of NAD^+ does not absorb radiation at 254 nm (the wavelength of detection) and therefore cannot be detected by this system. ATP is present in the assay from the activation procedure of pertussis toxin. It is highly charged and is not retained by the column; it is eluted at 4.0 min, after one column volume of buffer has passed through the column. The dodecamer and hexamer peptides bind to the reverse-phase column and are eluted in a 5-100 % methanol gradient as shown in Figure 4.8.

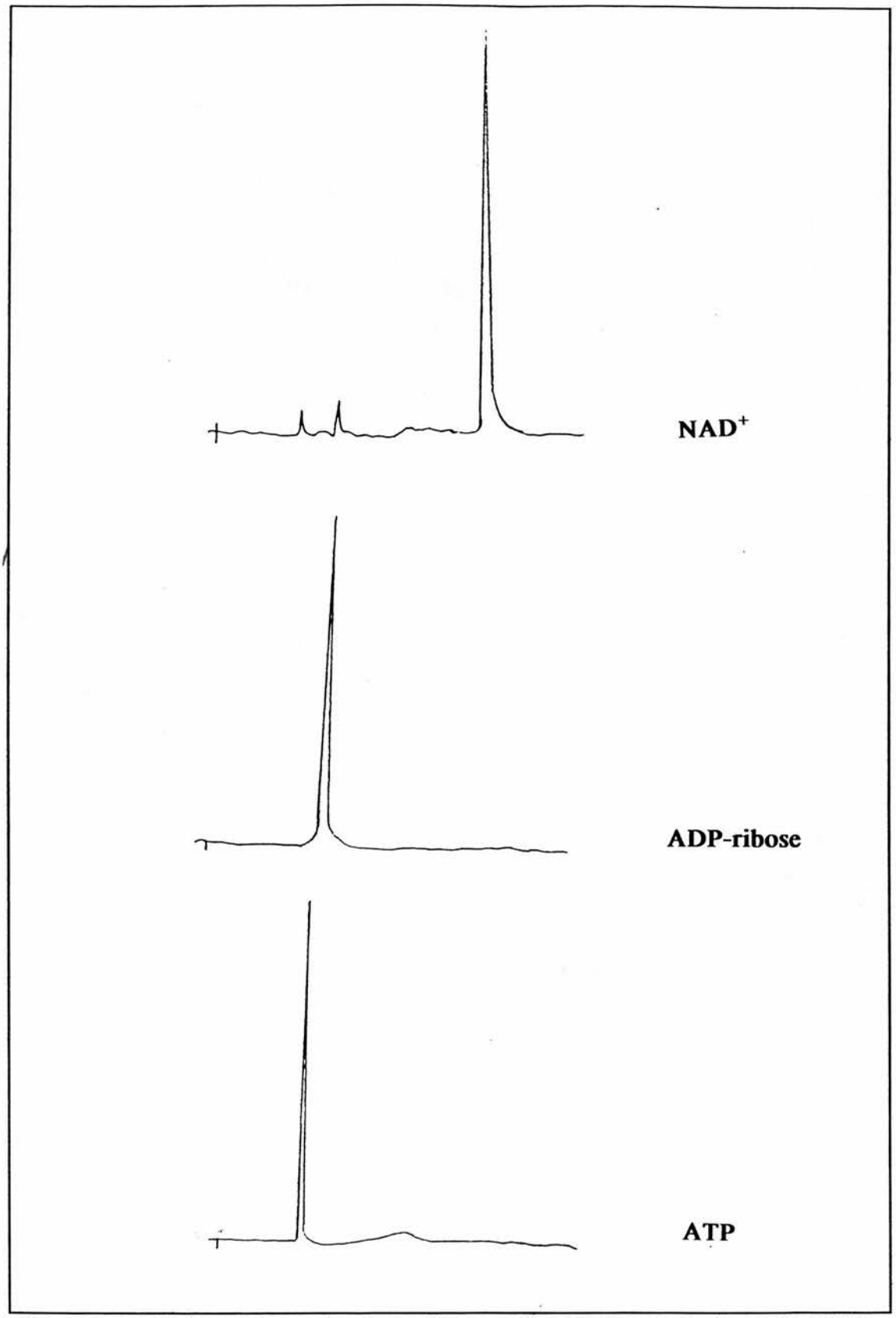


Figure 4.7 Elution of NAD^+, ADP-ribose and ATP Standards from ODS-HYP-2734, Reverse Phase HPLC. Elution achieved at 1 mL/min and detected at 254 nm 0.25 cm/min (chart recording).

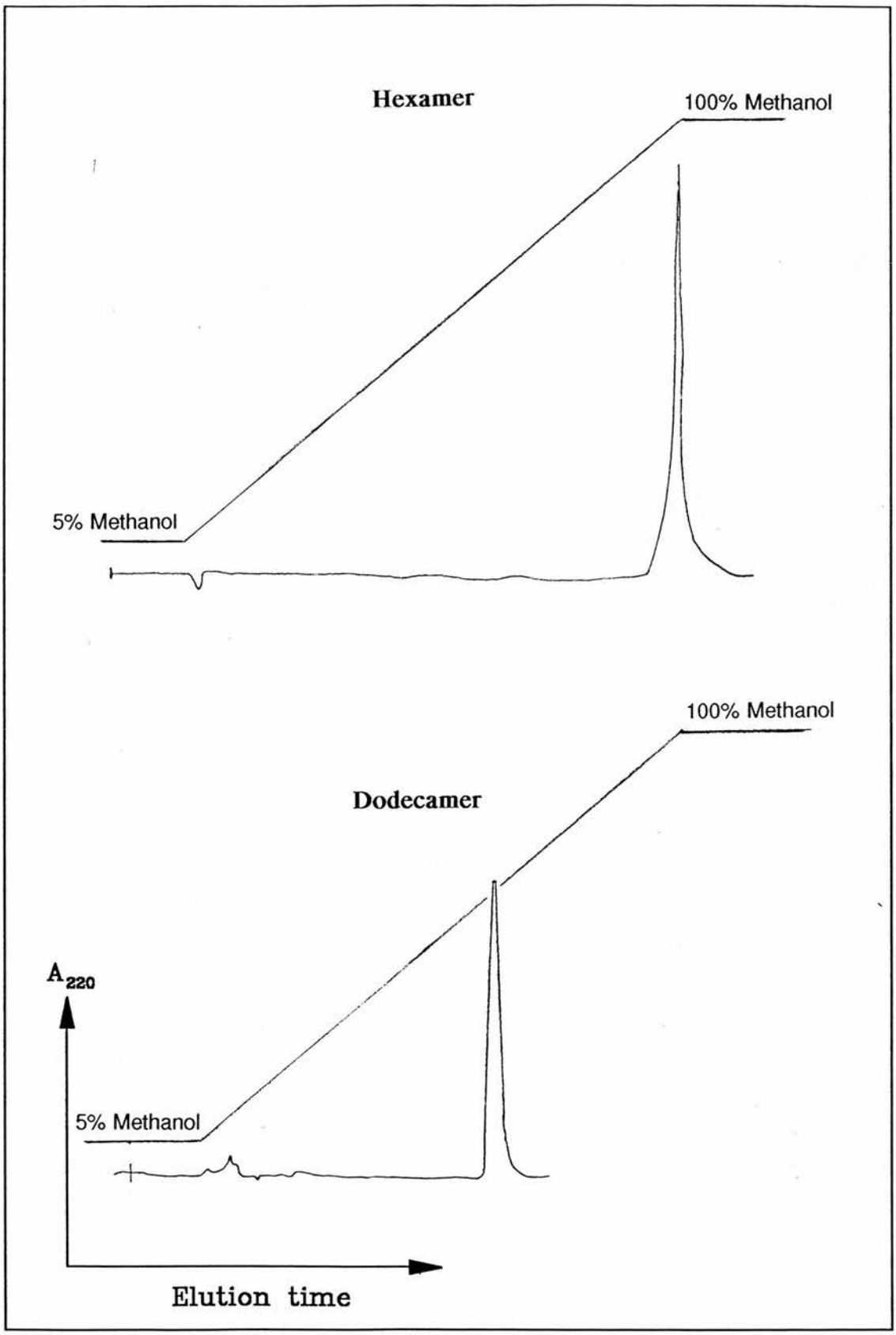


Figure 4.8 Elution of Hexamer and Dodecamer Peptides from Reverse Phase Column. 5-100% methanol gradient was run and the peptide was detected at 220 nm.

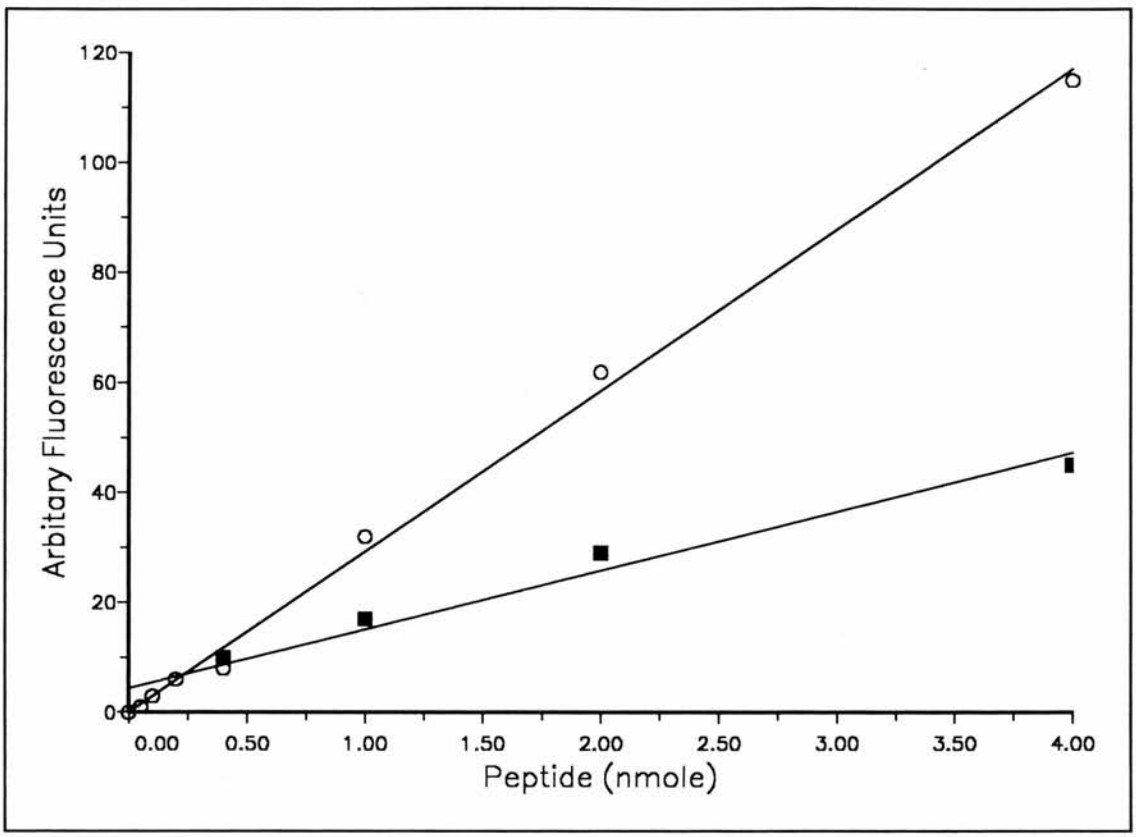


Figure 4.9 Dose Response Curves of Hexamer (■) and Dodecamer (○) Peptides With OPA reagent

1mg each of the dodecamer and hexamer peptide was incubated with pertussis toxin overnight at 30 °C. The products of the assay were separated on the reverse phase column and the elution profiles at 254 nm are shown in Figure 4.10 (a and b) and Figure 4.11 (a and b). Fractions were collected and tested for the presence of [^{3}H] label, by scintillation counting, and of peptide by fluorescence detection using OPA reagent. These data are shown in Figure 4.12 and Figure 4.13 for the dodecamer and the hexamer peptide respectively. The reaction of OPA with peptides is dependent upon the N terminal amino acid. It does not react with cysteine and reacts poorly with lysine residues; the N-terminal amino acid of the hexamer. The dose reponse curves of each of the peptides with OPA is shown in Figure 4.9. The minimum amount of dodecamer detectible is 1 nmole, which is equivalent to 0.3 % of the total amount of peptide added to the ADP-ribosylation assay. The minimum amount of hexamer detected was 2 nmole, which is equivalent to 0.3 % of the total peptide added to the assay. Assuming that each peptide molecule is modified by one ADP-

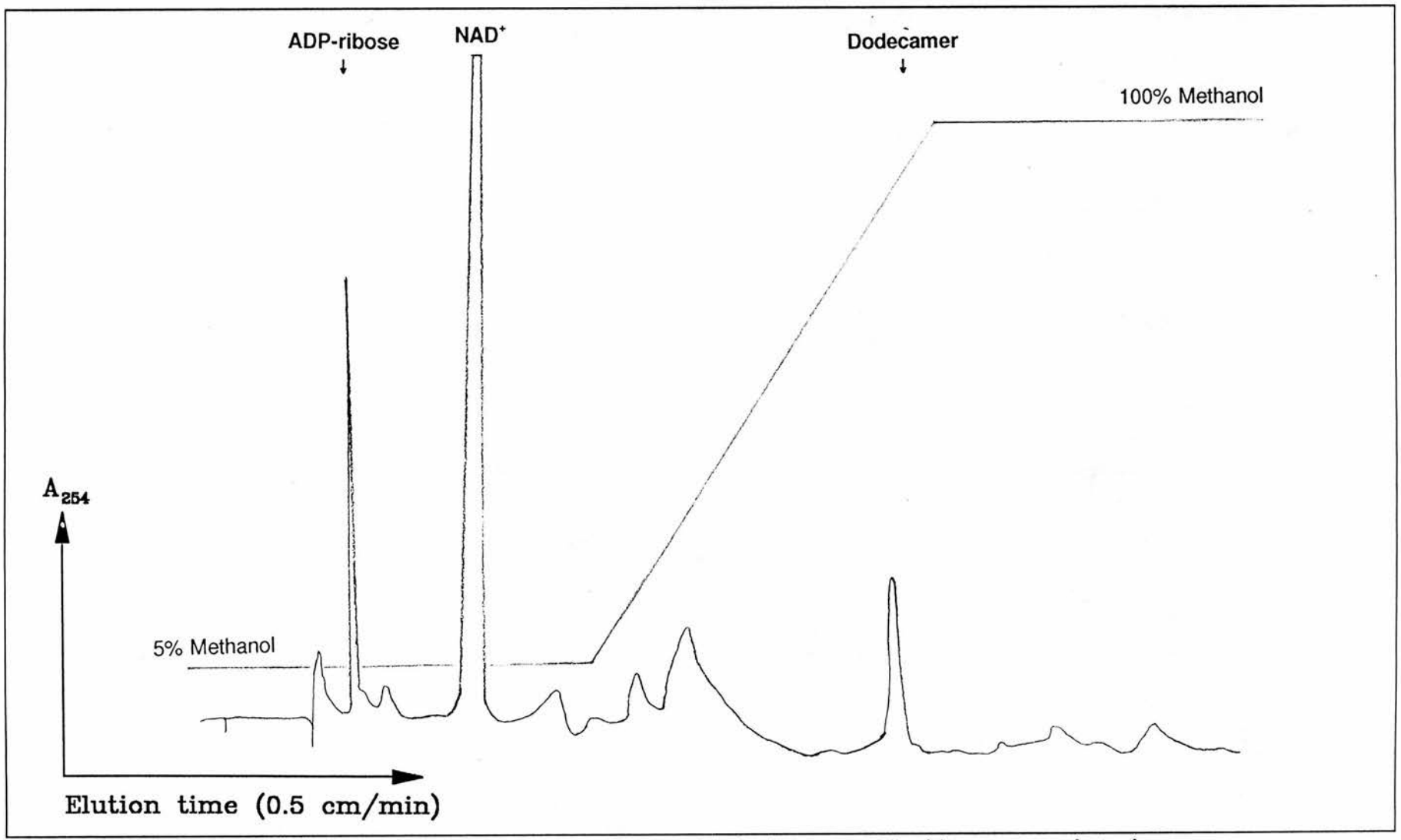


Figure 4.10(a) HPLC trace of the products of the incubation with dodecamer and without pertussis toxin.

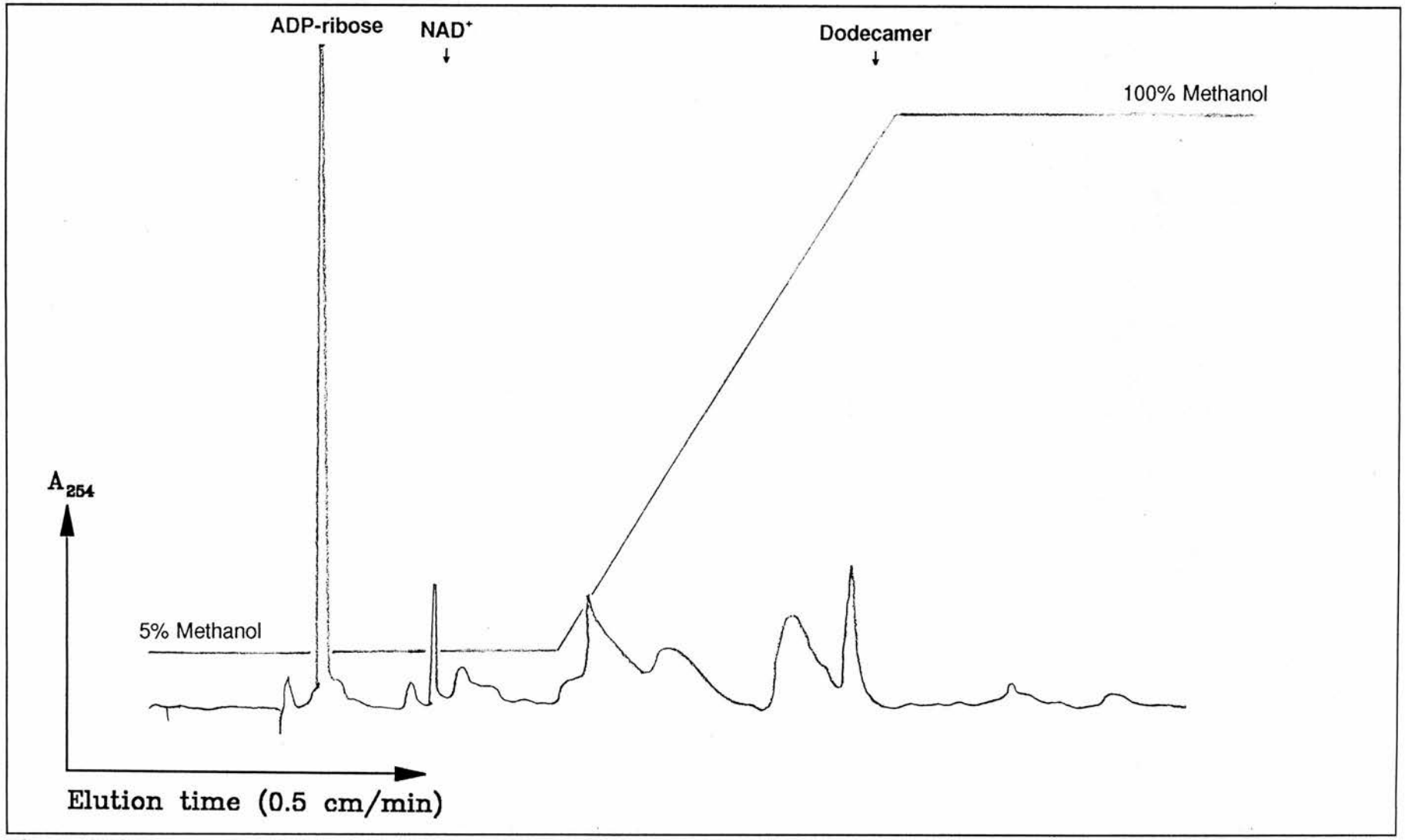


Figure 4.10(b) HPLC trace of the products of the incubation with dodecamer and pertussis toxin.

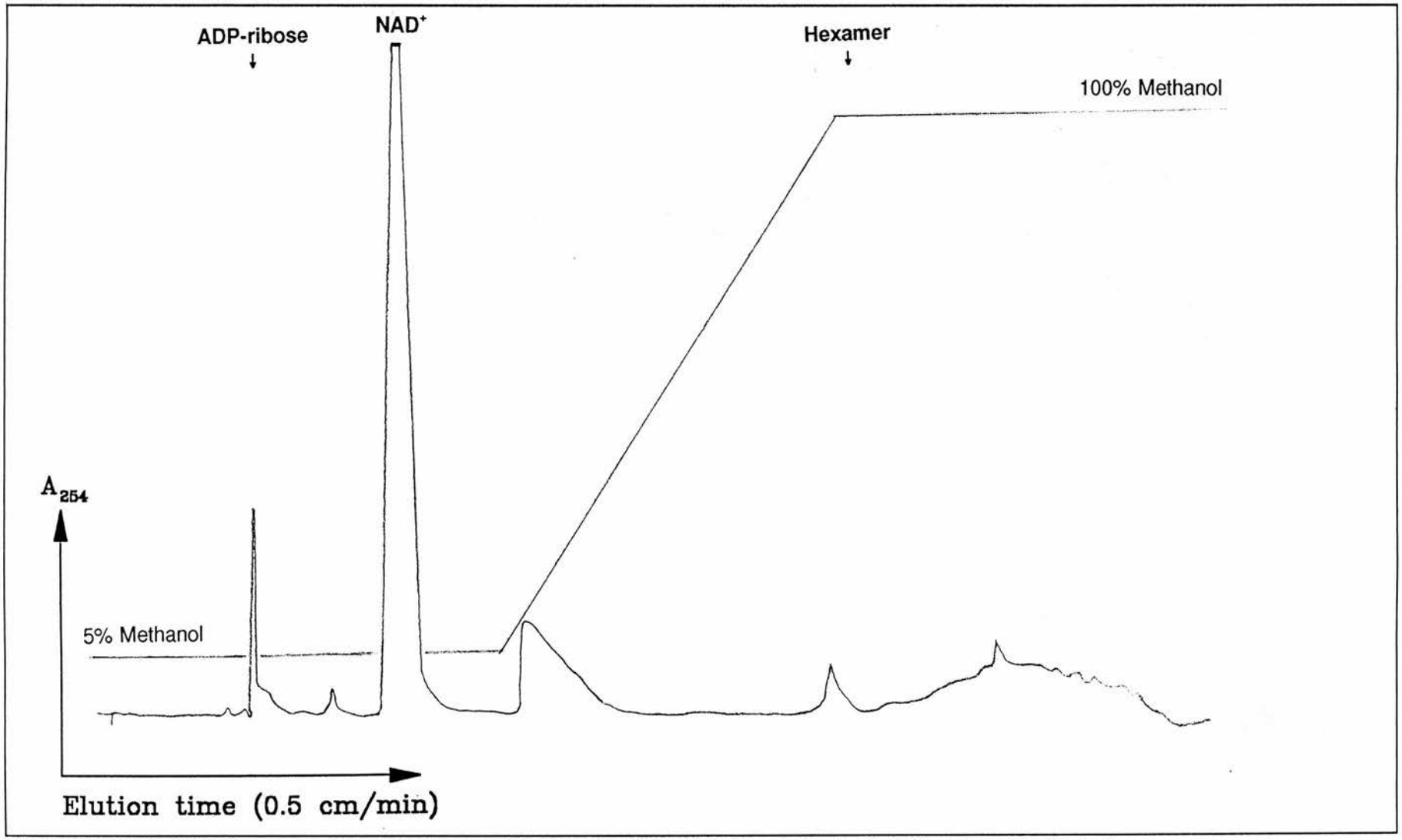


Figure 4.11(a) HPLC trace of the products of the incubation with hexamer and without pertussis toxin.

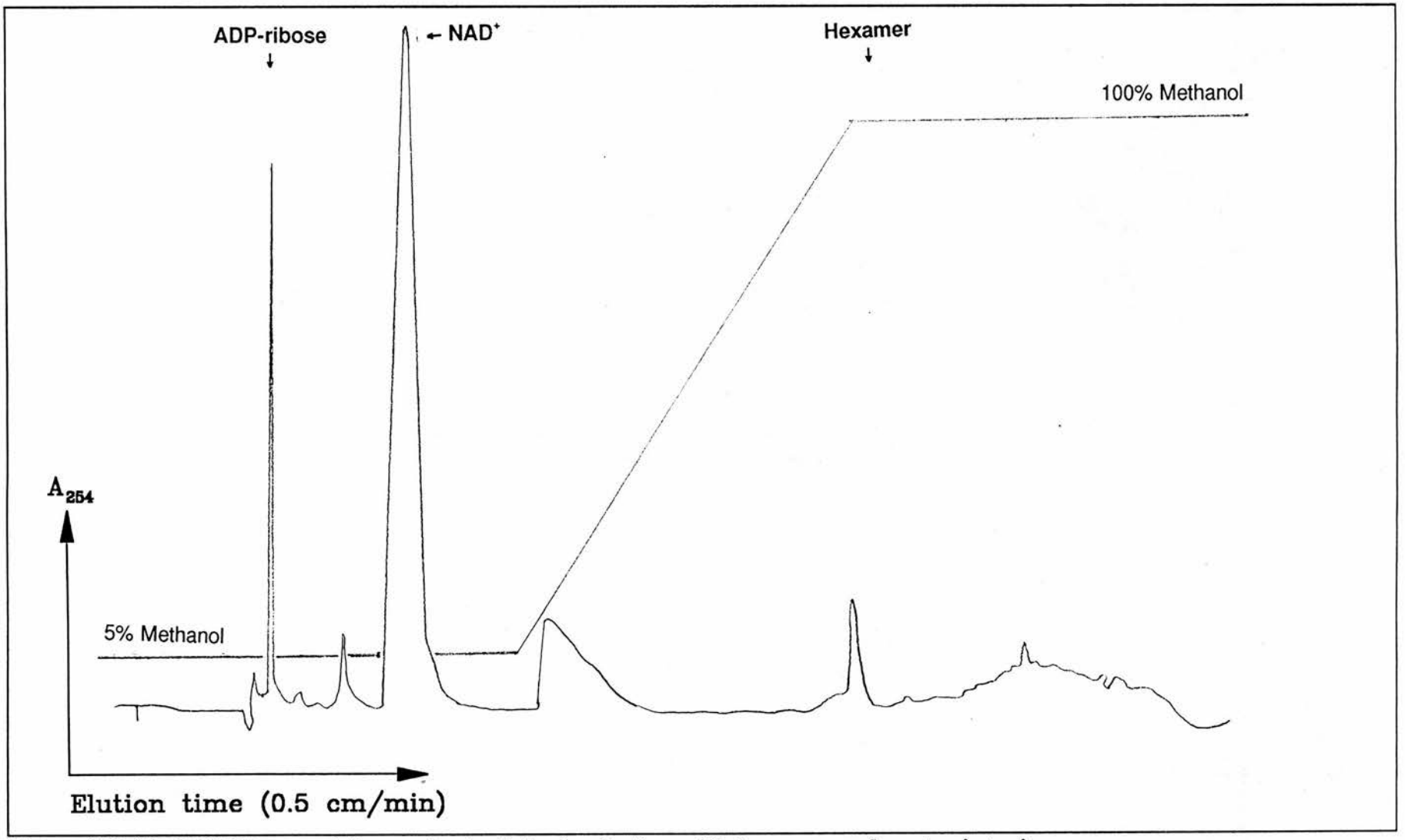


Figure 4.11(b) HPLC trace of the products of the incubation with hexamer and pertussis toxin.

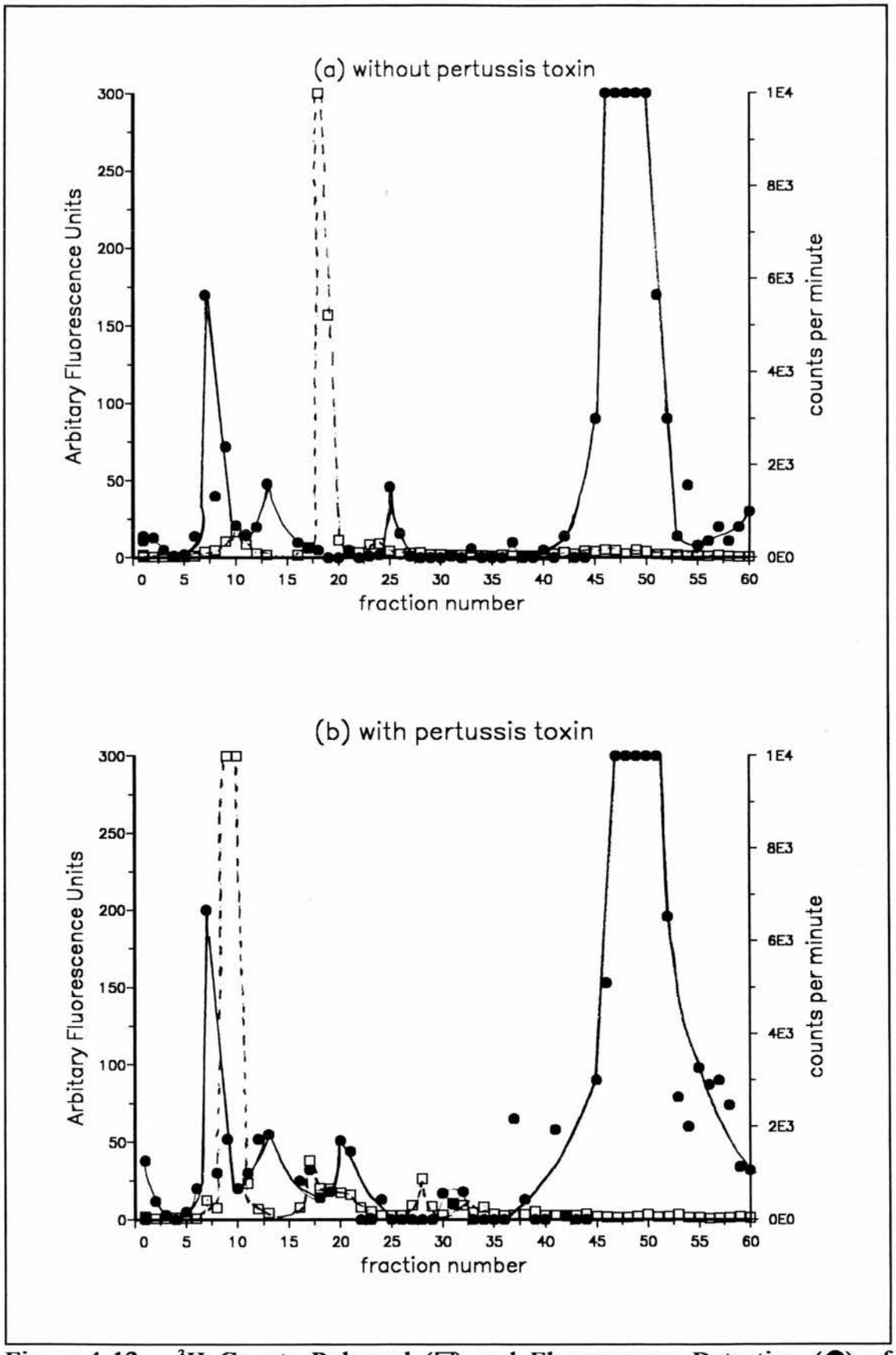


Figure 4.12 ^{3}H **Counts Released (□) and Fluorescence Detection (●) of Hexamer Peptide in the Eluate from reverse phase HPLC.**

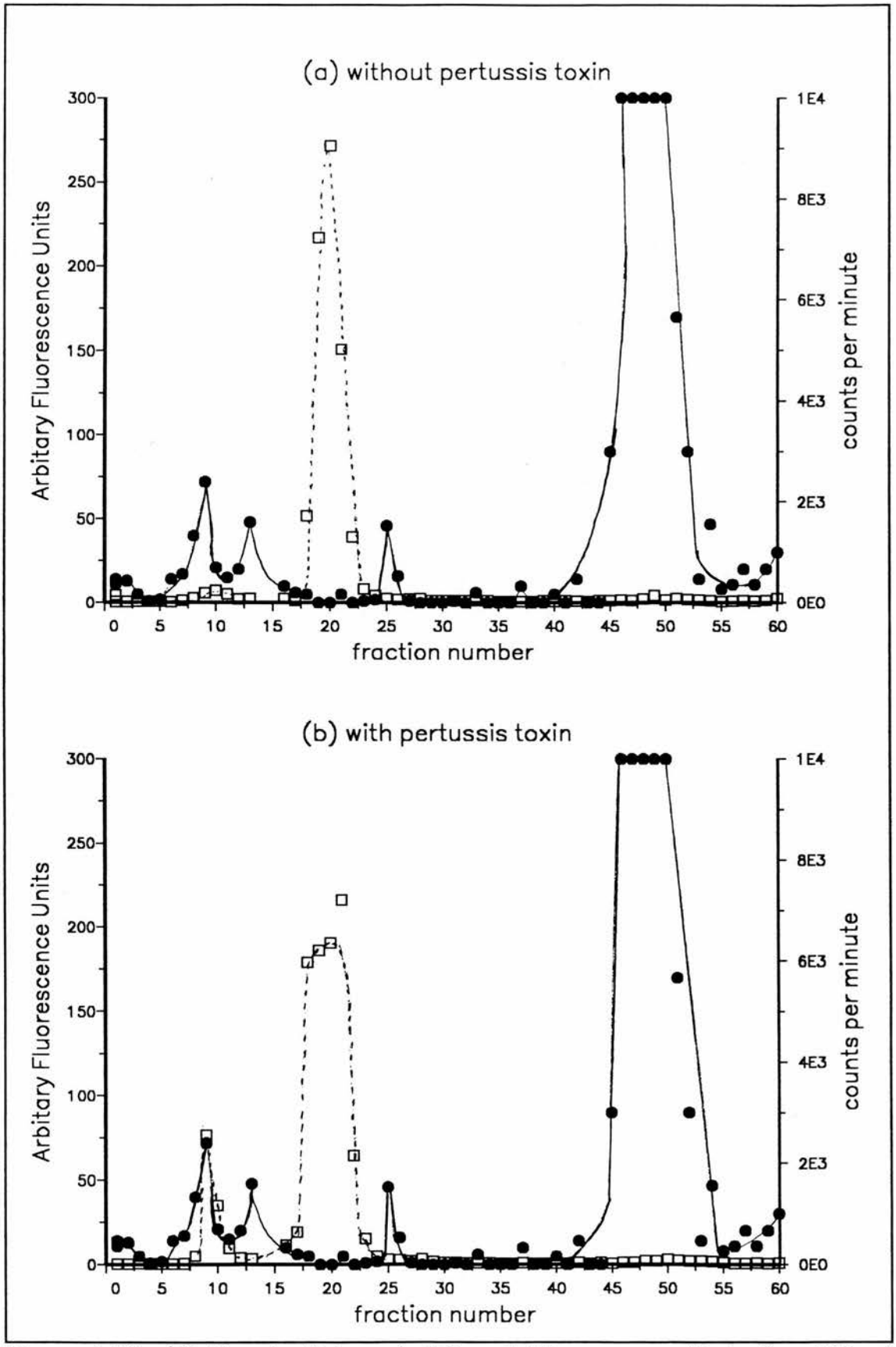


Figure 4.13 ^{3}H **Counts Released (□) and Fluorescence Detection (●) of Dodecamer Peptide in the Eluate from reverse phase HPLC.**

ribose molecule then for 1 nmole of peptide, 2000 counts per minute would be detected (2 cpm/pmol). Examination of Figure 4.12 shows that in the presence of pertussis toxin and dodecamer peptide 90 % of the NAD^+ is hydrolysed to ADP-ribose during the time course of the reaction. A peak of radioactivity is observed in fractions 24 and 25 (about 1000 cpm) in the control incubation without pertussis toxin. A similar sized peak is observed in the presence of pertussis toxin but has shifted in elution time and is seen in fraction 28. Neither of these fractions were fluorogenic on reaction with OPA and further characterisation of the nature of these peaks was not pursued. A very low level of counts was observed in fractions 31-34 in the presence of pertussis toxin, which were not evident in the control. A small increase in the amount of fluoresence detected above background was detectible in these fractions and was not evident in the control. This increase was at the limit of sensitivity of the assay and could not be measured reliably. The total number of counts associated with this fraction is equivalent to 500 pmole of ADP-ribose. Assuming a ratio of 1:1 ADP-ribose incorporated into the peptide then this is equivalent to 0.07 % of the total peptide. Whether this product is ADP-ribose-dodecamer is doubtful. The product was not detected after shorter incubation times (3 - 8 h) or for incubations with ten fold less NAD^+ (0.1 mM instead of 1 mM). It is possible that it was generated non-enzymatically in the presence of excess ADP-ribose, generated by the NAD glycohydrolase activity of pertussis toxin, during the prolonged incubation overnight.

Examination of Figure 4.11 shows that in the presence of hexamer and pertussis toxin NAD glycohydrolase was inhibited. Only 10 % of the total NAD^+ has been hydrolysed under conditions where complete hydrolysis may have been expected (as seen in incubations with the dodecamer peptide). No putatative ADP-ribose-hexamer containing fractions were detected. The ability of the peptides to inhibit the NAD glycohydrolase assay was tested further by adding 0-0.5 mM concentrations of peptide to the NAD glycohydrolase assay. The results are shown in Figure 4.14 and Figure 4.15 for the dodecamer and hexamer peptides respectively. 50 % inhibition of the NAD'ase activity was observed at a concentration of 0.2 mM for both peptides.

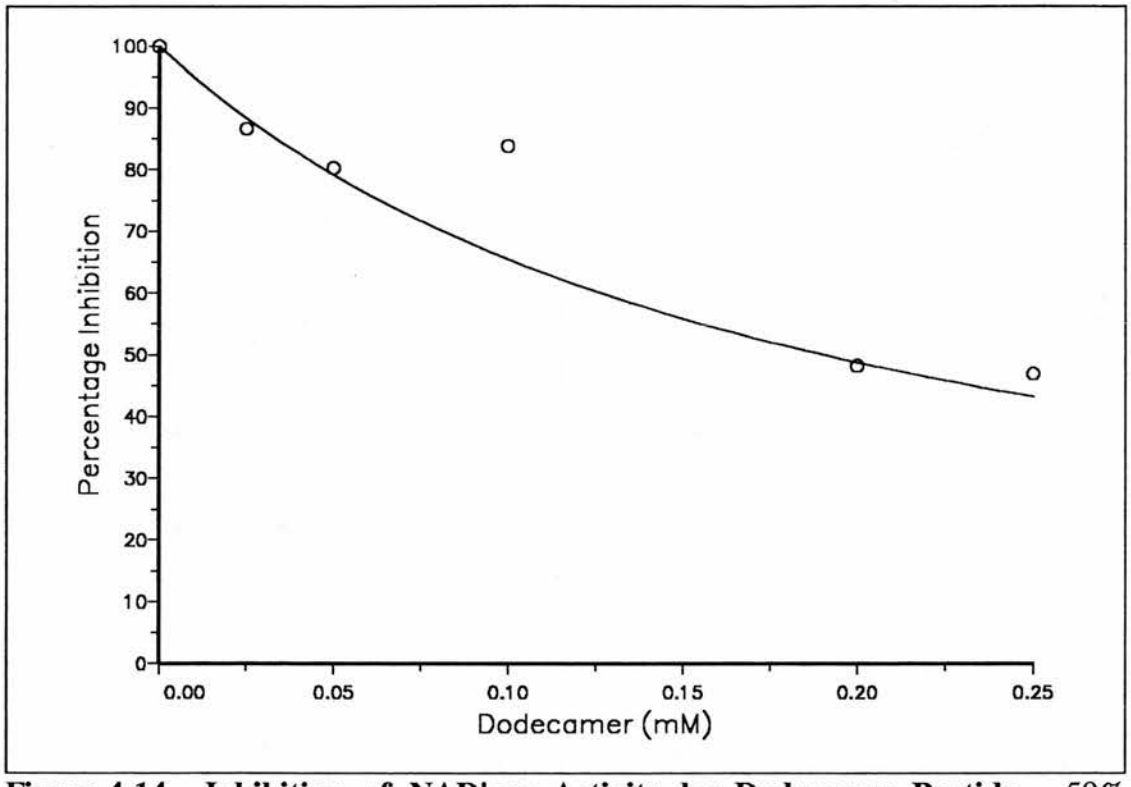


Figure 4.14 Inhibition of NAD'ase Activity by Dodecamer Peptide. 50% inhibition is observed at 0.19 mM concentration of peptide.

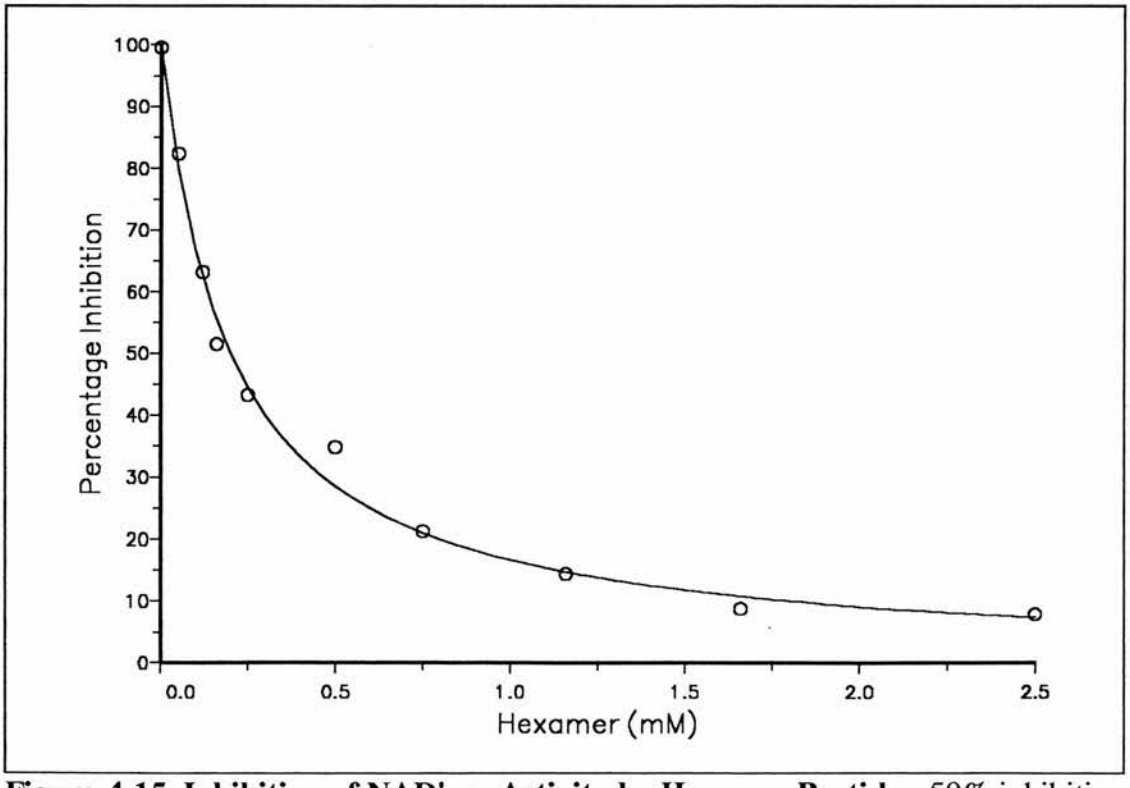


Figure 4.15 Inhibition of NAD'ase Activity by Hexamer Peptide. 50% inhibition is observed at 0.2 mM concentration of peptide.

Table 4.1 Summary of Alternative Substrates for Pertussis Toxin

SUBSTRATE	K_m (mM)	V_{max} relative	V_{max}/K_m	E_o' [149]
Dithiothreitol	9±2.5	1	35	-0.33
cysteine	109±30	1.72	0.48	-0.22
S-carboxymethyl-cysteine	-	-	-	-
cysteine-methylester	116±25	1.46	0.42	-0.22
cys-gly	90±19	1.75	0.44	-0.22
glutathione	20±3	0.81	1.5	-0.24
mercapto-pyridine	>500	-	-	
mercapto-imidazole	>500	-	-	-0.06
hexamer	0.2	50% inhibition		
dodecamer	0.19	50% inhibition		

4.2.3 Discussion

The small molecule free thiol containing compounds tested all showed apparent substrate kinetics. In the table 4.1 above the K_m value gives a measure of the affinity of the substrate for pertussis toxin and V_{max}/K_m gives a measure of the specificity of the substrate. DTT showed the highest specificity of all the substrates tested. It had the highest affinity (K_m=5 mM) and a maximal rate of 33 pmol.min^{-1}. The cysteine like substrates containing a free thiol group showed similar specificity values to each other (K_m=100 mM, V_{max} 55 pmol.min^{-1}). This suggests that neither binding nor catalysis were sensitive to the negative charge on the carboxyl group. Larger structures such as glutathione showed a five fold increase in affinity compared to cysteine, but V_{max} was reduced such that the specificity of the substrate was low compared to DTT. No substrate affinity was found at all for the hexamer and dodecamer peptides, although tight binding affinity was suggested from the observed inhibition of NAD'ase (50 % inhibition at 0.2 mM). The aromatic thiol containing compounds had no effect on the NAD glycohydrolase activity of pertussis toxin. They were niether substrates, nor inhibitors of the toxin.

One interpretation of these data assumes that the apparent Michaelis-Menten kinetics observed is due to the thiols acting as acceptors for ADP-ribose and competing with water at the active site. The small thiol compounds are free to enter the active site of the enzyme and may dissociate readily. Maximal catalysis is observed. Glutathione binds more tightly than cysteine to the enzyme but is not quite the right conformation to readily accept ADP-ribose. Its presence at the active site blocks hydrolysis by water and reduced V_{max} are observed. This effect is taken to the extreme in the presence of hexamer and dodecamer peptide. Effective binding is achieved but no turnover of the substrate occurs.

It was disappointing that no effective substrate mimic was found. Although the C-terminal amino acid composition is important for ADP-ribosylation of $G_i\alpha$ it appears that it is not sufficient on its own to act as an acceptor. Perhaps a longer peptide is required to achieve the correct folding or possibly distant portions of the $G_i\alpha$ are essential for the correct protein protein interactions. Studies with α_s and α_i G protein chimeras confirm that the C-terminal portion is not sufficient for ADP-ribosylation by pertussis toxin[150]. It is also known that efficient ADP-ribosylation of

$G_i\alpha$ requires the interaction of the $\beta\gamma$ subunits[151]. It is perhaps not so surprising that the peptides were able to interact with the toxin, causing inhibition of the NAD glycohydrolase activity but fail to act as substrates for ADP-ribosylation.

Since this work was completed Graf *et al.*[152] have made a comprehensive study of the inhibition of ADP-ribosylation of $G_i\alpha$ by C-terminal synthetic peptides of 10-20 amino acids in length. The longer the peptide the greater the inhibition of ADP-ribosylation of G_i that was observed. This trend was not seen for the dodecamer and hexamer, in their ability to inhibit the NAD glycohydrolase assay. Interestingly, Graf *et al.* observed that the C20 peptide was ADP-ribosylated, and they developed an assay to determine the kinetics parameters for several C-terminal peptides.

Table 4.2 Some Peptide Substrates of Pertussis Toxin from Graf *et al.*[153]

PEPTIDE	COMPOSITION	K_m (μM)	V_{max} (pmol/min/mg PTX)
C-12 synthesised	I I K N N L K D C G L F		
C-15 G_{i1} and G_{i2}	T D V I I K N N L K D C G L F	480	15
C-15 G_{i3}	T D V I I K N N L K E C G L Y	120	42

The assay involved the TCA precipitation of the peptides onto glass fibre filters, and the amount of [^{32}P] incorporated was counted using a scintillation counter. The reaction conditions were sub-saturating for NAD glycohydrolase activity (20 μM NAD^+) and carried out for 3 h at 4 °C. The use of low concentrations of [^{32}P]NAD^+ made this assay system 100 times more sensitive than the HPLC detection system used in the experiment described above. However the results should be treated with some caution since non-specific labelling may be detected using the filter binding

assay, and direct evidence for the modification of the peptide by ADP-ribose was not demonstrated. The data showed that small changes in the amino acid composition could produce a four fold difference in the affinity of pertussis toxin for peptides of the same length. The C- 15 amino acids of $G_{i1/2}\alpha$ have K_m value of 480 μM, whereas the C-15 amino acids of $G_{i3}\alpha$, in which asp-250 is changed to glu and phe-254 is changed to tyr, have a K_m 120 μM.

From table 4.2 the reasons why the dodecamer and hexamer synthesised were poor substrates for pertussis toxin become apparent. Unluckily, the dodecamer is too short and corresponds to the sequence which has a reduced substrate specificity for pertussis toxin.

An alternative explanation for the apparent substrate kinetics observed for the small thiol containing compounds may be based on the extent of reduction of the toxin.

$$E_i \underset{k_r}{\overset{k_f\,[RSH]}{\rightleftharpoons}} E_a \xrightarrow{k_{cat}} \textbf{Products}$$

This model proposes that when the enzyme is incubated with reducing reagent [RSH], the interconversion rate of active and reduced toxin (E_a) and inactive oxidised toxin (E_i) is described by the reversible equilibrium reaction above. In the absence of reductant all of the enzyme will be in the inactive form. Reductant is required to maintain the enzyme in its active form. Removal of the reductant after activation will result in the rapid re-equilibration to the inactive form.

The total amount of enzyme E_o present is

$$E_o = E_i + E_a \qquad (1)$$

and the amount of active enzyme present in the steady state will be defined by

$$E_a = E_i k_f[RSH] - E_a k_r \qquad (2)$$

therefore, by substitution equation (2) in equation (1)

$$E_0 = E_a \left(1 + \frac{(1 + k_r)}{k_f [RSH]}\right) \qquad (3)$$

The rate of product formation obsereved, V_{obs} is defined by $E_a.k_{cat}$. Substituting the equation (3) into this formula shows that the rate observed will vary as a rectangular hyperbole with respect to the concentration of reductant.

$$V_{obs} = \frac{k_{cat}\, E_o\, [RSH]}{k_f [RSH] + (1 + k_r)} \qquad (4)$$

This is the same expression as the Michaelis Menten equation for which the data were a good fit. If the toxin is highly sensitive to oxidation and rapidly inactivates in the absence of high concentrations of DTT then the apparent substrate kinetics observed may be a function of activation. The K_m observed will be the concentration of reductant required to half maximally activate the toxin, i.e. the condition where half the enzyme is reduced ($[Ei]=[Ea]$ in the model). The concentration of reductant required to maintain the toxin in the half reduced state (K_m observed) will be dependent on how good a reductant is the compound. The standard electrode potential (E_o') is a measure of reducing potential; the lower the potential the better the reducing power. From the Nernst equation, the higher the standard electrode potential is for a compound then the more of that compound will be required to maintain a given potential. Figure 4.16 shows the plot of E_o' versus K_m observed for DTT, glutathione and cysteine and confirms the expected trend.

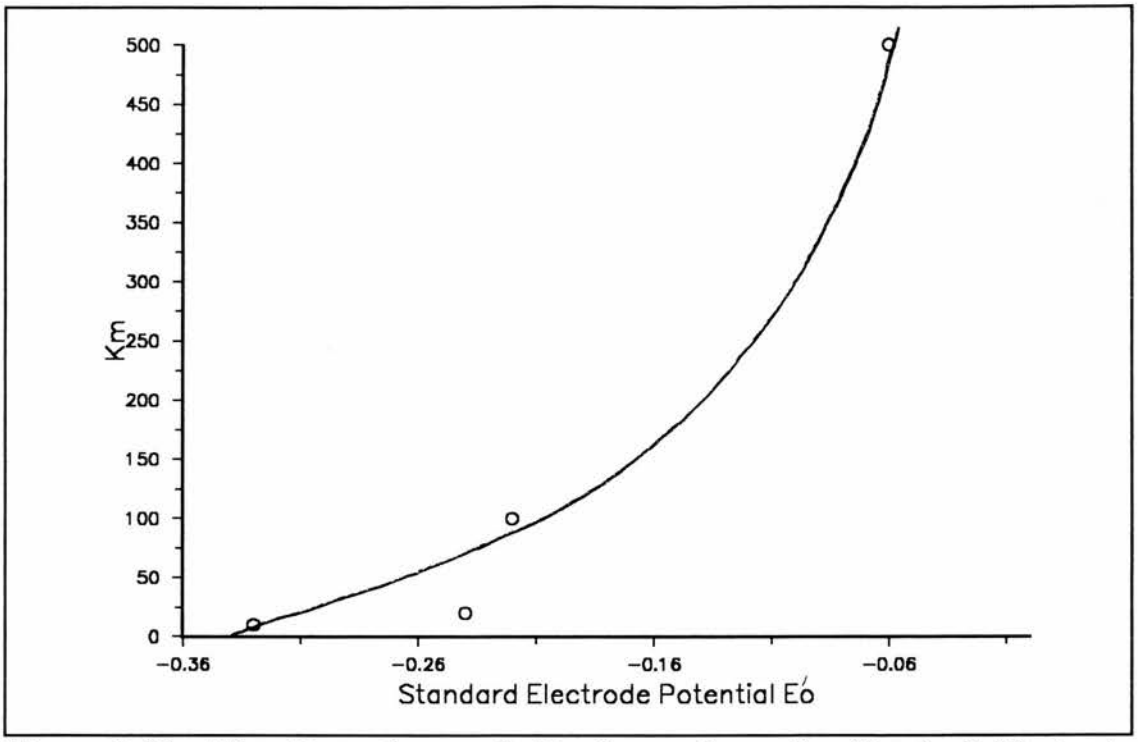


Figure 4.16 The Dependence of K_m observed on the Standard Electrode Potential of the Substrate

For this model to be true, then the equilibrium is in favour of the inactive form of the enzyme. High concentrations of reductant are required to push the equilibrium towards the active form of the enzyme and maintain the enzyme in the active form. Activation of pertussis toxin with 100 mM DTT and subsequent removal of the reductant by dilution results in less than maximal NAD'ase activity being observed, even when precautions have been taken to minimise oxidation reactions. The high concentrations of reductant required and the rapid inactivation of the enzyme in the absence of oxidants are unusual properties of a redox system. These factors and the fact that the V_{max} observed for each reductant tested was not the same, suggest that the apparent substrate kinetics observed are not simply a function of reduction of the enzyme.

In the above experiments the thiol compounds are probably functioning as both acceptors and activators of the enzyme. The assay measures nicotinamide release and does not give any indication of the proportion of ADP-ribose to ADP-ribose-acceptor generated. Clarification of the kinetic processes involved requires the development of an assay which differentiates ADP-ribose and ADP-ribose-acceptor directly. This is beyond the scope of this thesis.

4.3 Kinetic Properties of Cysteine Dependent NAD'ase

4.3.1 Time Course

A modification of the NAD'ase assay described by Moss and Vaughan was followed as described in section 2.3. The assay allows single time point measurements. For valid kinetic analysis, conditions must be found where NAD^+ turnover is limited to 20% of the total and is linear over the time course of the reaction.

4.3.1.1 Results

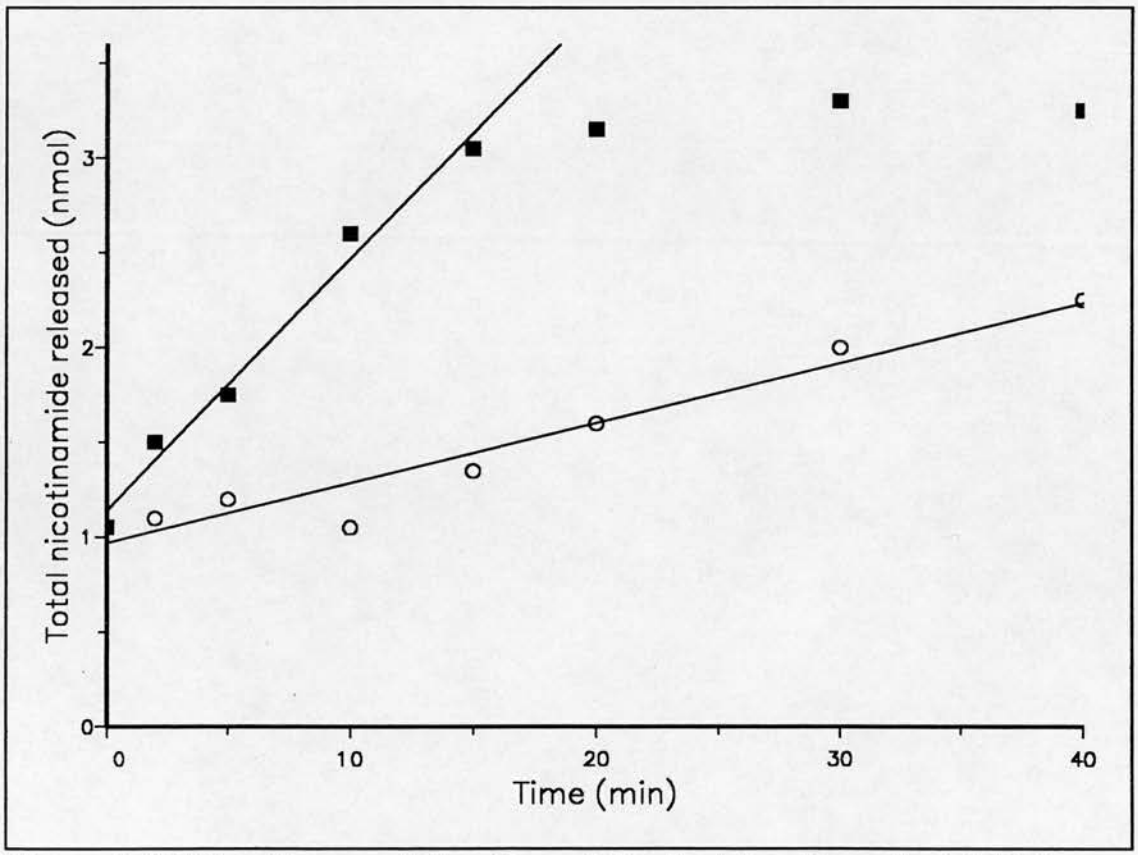


Figure 4.17 Time Course of Reaction of the Cysteine Dependent NAD'ase
The amount of nicotinamide released at given time intervals was measured in the presence (■) and absence (○)of enzyme

The NAD'ase assay was set up to contain saturating levels of subtrates. At given time intervals the reaction was stopped by application of the reactants onto the DOWEX resin. The amount of nicotinamide released was calculated and is shown in Figure 4.17. 30% of the total NAD^+ is used up in 30 minutes and is linear over

this time period. Extended incubation times, beyond 30 minutes did not release any more nicotinamide. This could be due to product inhibition or inactivation of the enzyme, which is very labile (see section 3.4). Non-enzymatic breakdown of NAD^+ over the time course is also shown. 8% of total NAD^+ at time zero increased to 15% over the time course of the experiment. Some hydrolysis of the NAD^+ stock had occurred on storage at pH 5.0, 4°C. The NAD'ase assay is performed at neutral pH and in the presence of phosphate ions and thiol reagents, all of which are known to accelerate the hydrolysis of the glycosidic linkage between ADP-ribose and nicotinamide[153].

4.3.2 pH Optima

4.3.2.1 Method

The effect of pH on the maximal rates of NAD'ase activity were studied. 1 M stock solutions of monobasic potassium phosphate, dibasic potassium phosphate, sodium citrate, Tris base and 10 M HCl were made. The stock solutions were mixed together to give the required pH (calculated from the Henderson-Hasselbach equation). Phosphate buffer pH 6.0 - 8.0, citrate phosphate pH 5.0 - 6.6, Tris-HCl pH 7.4 - 9.0 were checked using a pH meter (Corning). NAD^+-glycohydrolase reactions were set up to contain 50 mM prepared buffer, 100 mM cysteine (made fresh and adjusted to neutral pH), 100 μM [^{3}H]NAD^+ and activated pertussis toxin or cysteine dependent NAD'ase. The enzymes were dialysed against low ionic strength buffer and control incubations containing an equal volume of dialysis buffer (or of activation mix for pertussis toxin) for each enzyme was performed. The reaction was stopped by the addition of 0.85 mL ice cold 50 mM Tris-HCl pH7.4. This buffers the incubation mixtures, such that the binding properties of NAD^+ to the ion exchange column are not effected. The amount of released nicotinamide produced was detected by scintillation counting.

4.3.2.2 Results

Figure 4.18 and Figure 4.19 show the pH profiles obtained under the assay conditions described for cysteine dependent NAD'ase and pertussis toxin respectively. Background and enzymatic rates of nicotinamide released are shown. As expected[154],

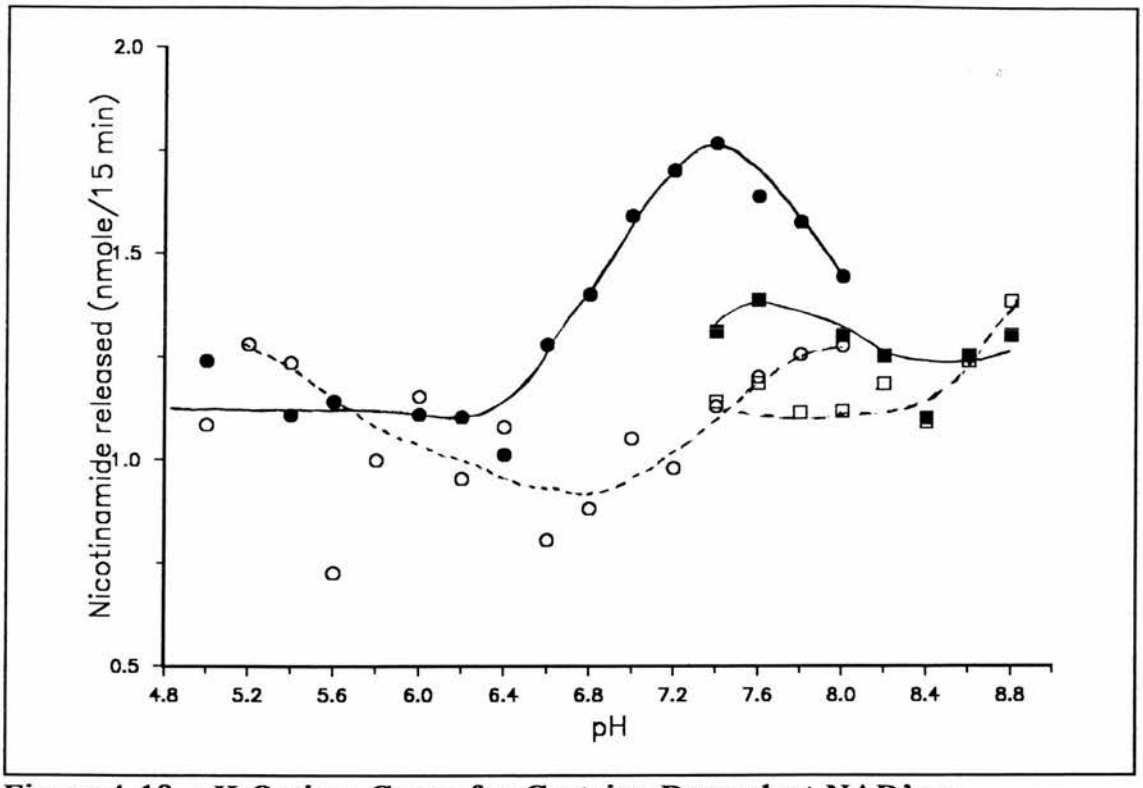


Figure 4.18 pH Optima Curve for Cysteine Dependent NAD'ase.
Incubations containined phosphate buffer with (●) and without (○) enzyme or Tris bufffer with (■) and without (□) enzyme.

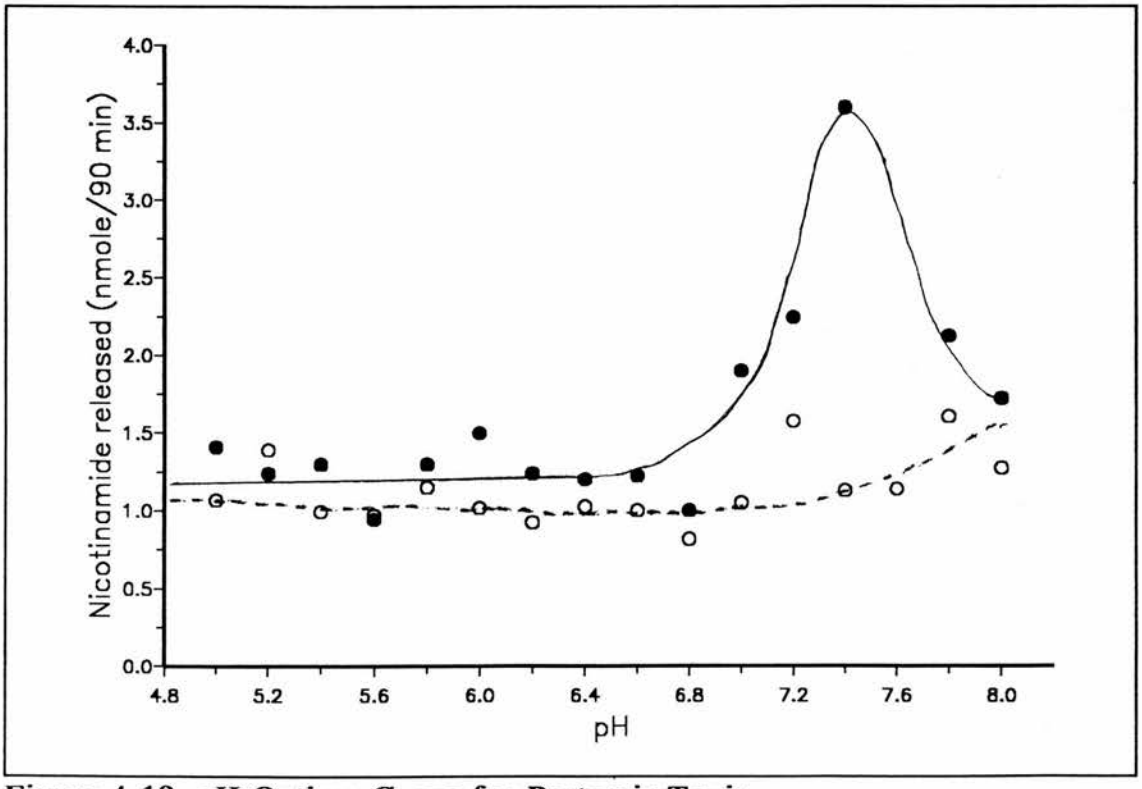


Figure 4.19 pH Optima Curve for Pertussis Toxin.
Incubations containined phosphate buffer with (●) and without (○) enzyme

at neutral and alkali pH the rate of backgound hydrolysis increased; this was most prominently observed for incubations with pertussis toxin which were run for 90 min. Both the endogenous enzyme and pertussis toxin show a characteristic bell shaped curve with pH optima pH7.4 - 7.6 at saturating concentrations of substrates. Control incubations were set up to check that denaturation of the enzymes at the high and low pH tested did not occur. The enzymes were incubated in buffer at pH 5.5 or pH 8.6 (15 min for the endogenous enzyme and 60 min for pertussis toxin) and then were assayed at maximal conditions (pH 7.4) and compared to untreated enzyme. No loss in activity was observed as a result of these pre-treatments, confirming that the change of activity observed over the range of pH tested was not due to denaturation of the enzymes.

Potassium phosphate buffer appears to activate the endogenous enzyme. Two fold higher activity was observed in the presence of phosphate compared to Tris-HCl at the same pH. Half maximal activity observed on the descending portion of the bell shaped curve for the cysteine dependent NAD'ase is observed at pH 8.1; this is close to the pK_a of the thiolate ion of free cysteine. The thiolate ion is a highly reactive nucleophile and at neutral pH values and above cysteine rapidly oxidises to cystine[154]. This modification of the acceptor substrate may account for the rapid loss in apparent activity.

4.3.3 NAD^+ Dose Response Curve

4.3.3.1 Method

NAD'ase activity was assayed as described in section 2.3. Incubations were set up containing 50 mM potassium phosphate, pH 7.4 and NAD^+ in the range 0 - 0.25 mM. Assays containing low concentrations of [^{3}H]NAD^+ (below 20 μM) were performed using a high specific activity [^{3}H]NAD^+ stock solution (1 Ci/mmole, 10 cpm/pmole) in order that significant counts could be detected. Pertussis toxin assays were performed in the presence of 250 mM DTT. Endogenous cysteine dependent NAD'ase assays were performed in the presence of freshly prepared 100 mM cysteine. The reactions were started by the addition of enzyme.

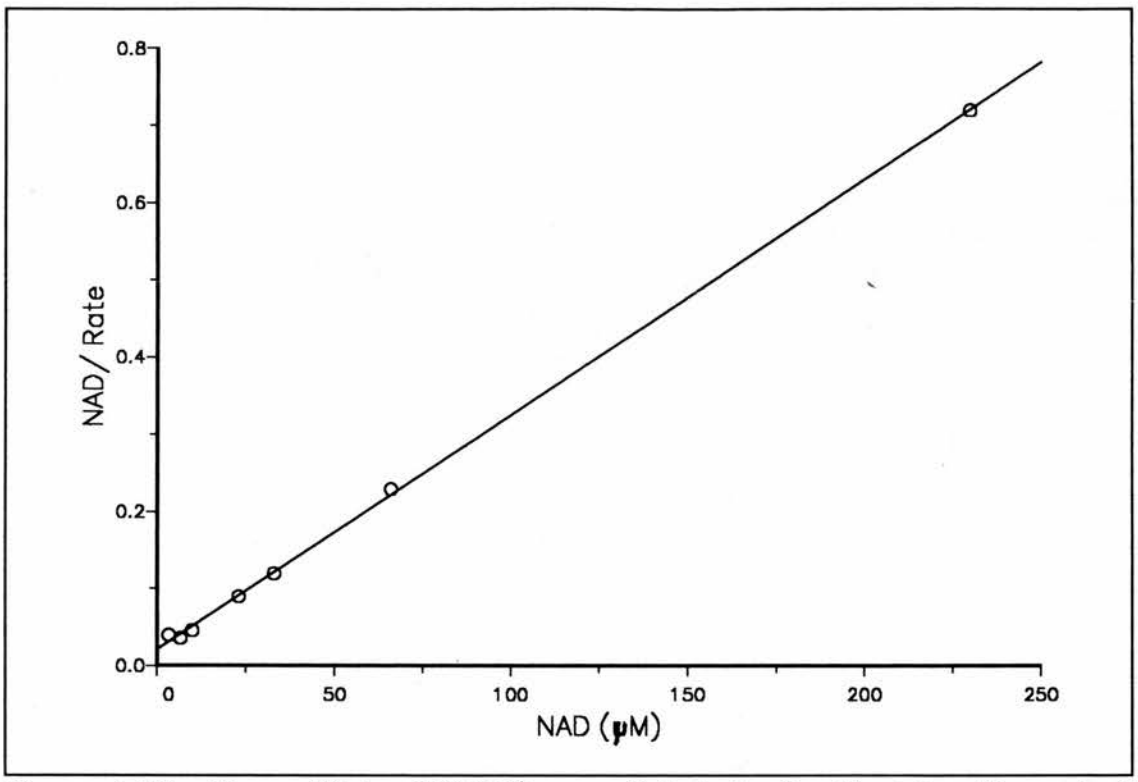


Figure 4.20 Hanes Plot of NAD^+ as a Substrate for Cysteine Dependent NAD'ase. K_m of 8 μM and V_{max} of 300 pmol·min^{-1} was estimated.

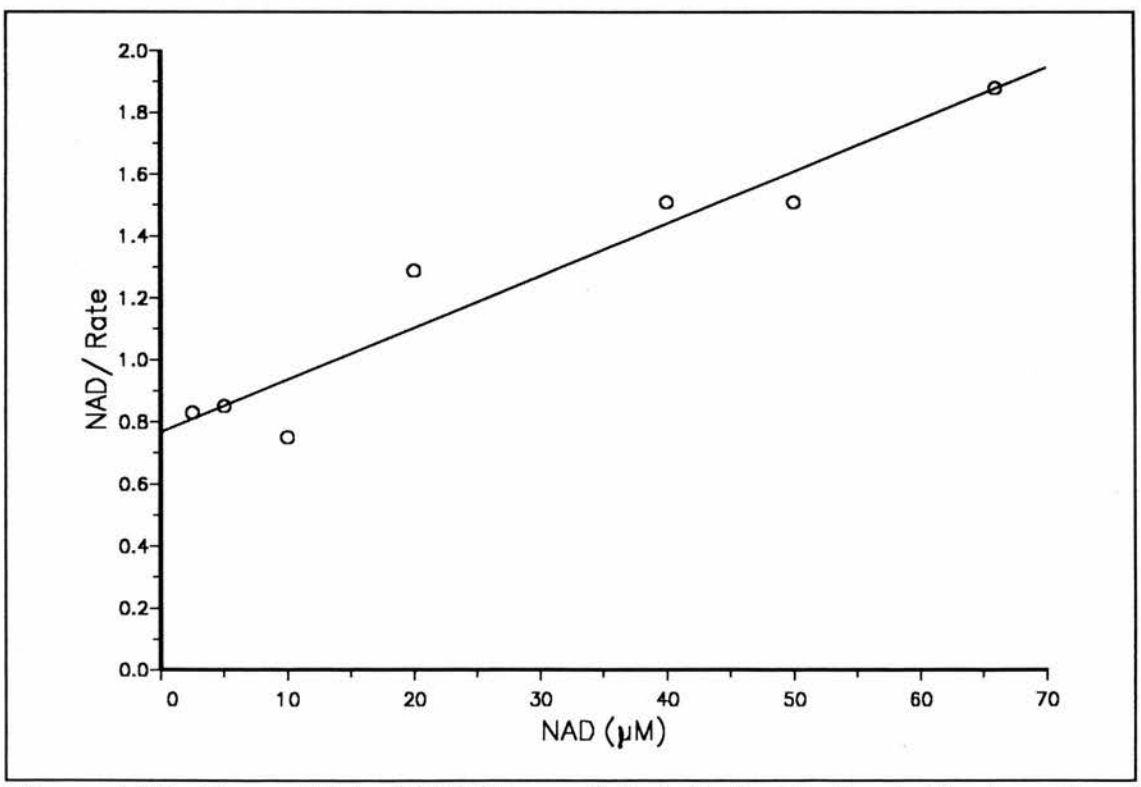


Figure 4.21 Hanes Plot of NAD^+ as a Substrate for Pertussis Toxin. K_m of 30 μM and V_{max} of 37.5 pmol·min^{-1} was estimated.

4.3.3.2 Results

The dose response curves obtained were fitted to the Michaelis-Menten equation using non-linear least squares curve fitting (see section 2.3.3). The Hanes plot of the data are shown in Figure 4.20 and Figure 4.21 for cysteine dependent NAD'ase and pertussis toxin respectively. A K_m of 30 μM and V_{max} of 37.5 pmol.min^{-1} was found using this method for pertussis toxin and a K_m of 8 μM and V_{max} of 300 pmol min^{-1} was found for the endogenous cysteine dependent activity.

4.3.4 Cysteine: A Substrate of Cysteine Dependent NAD'ase

In chapter three it was seen that the enzyme was inactive in the absence of cysteine. Here the affinity of cysteine for the enzyme and its ability to act as an acceptor for ADP-ribose was tested.

4.3.4.1 Method

NAD glycohydrolase assays were set up containing 50 mM potassium phosphate, pH 7.4 and 100 μM (^{3}H-nicotinamide)NAD$^+$. A 1 M stock solution of cysteine (free acid) was prepared fresh and neutralised with sodium hydroxide. All solutions were degassed to minimize oxidation reactions. Cysteine was added in the range 0 - 250 mM to the assay. The reaction was started by the addition 100 ng cysteine dependent NAD'ase and was incubated for 20 min at 30°C.

The reaction was repeated at saturating conditions of (^{3}H-Adenine)NAD$^+$ (100 μM) and cysteine (100 mM). After incubating for 30 min the reaction was stopped by cooling on ice. The cysteine dependent NAD'ase was removed by filtration through a 10,000 *Mr* cut-off membrane and the filtrate was applied to the reverse-phase HPLC column as described in section 2.4. The products of the NAD'ase assay were separated on the column by isocratic elution in 10 mM ammonium phosphate buffer, pH 3.5, 10 % methanol. 0.5 mL were collected and the amount of ^{3}H label contained in each fraction was measured by scintillation counting.

A putatative ADP-ribosyl-cysteine product was collected. To confirm the presence of an ADP-ribosyl cysteine linkage the product was treated with snake venom phosphodiesterase (from *Crotalus durissus terifficans*) following the

methodology of Lobban and van Heyningen[145]. The product was incubated with 20 μg/mL of phosphodiesterase in 50 mM potasium phosphate pH 7.5 for 5 h at 37 °C. The enzyme was removed by filtration through a 10,000 *Mr* cut-off membrane and the filtrate was applied to the reverse phase column.

4.3.4.2 Results

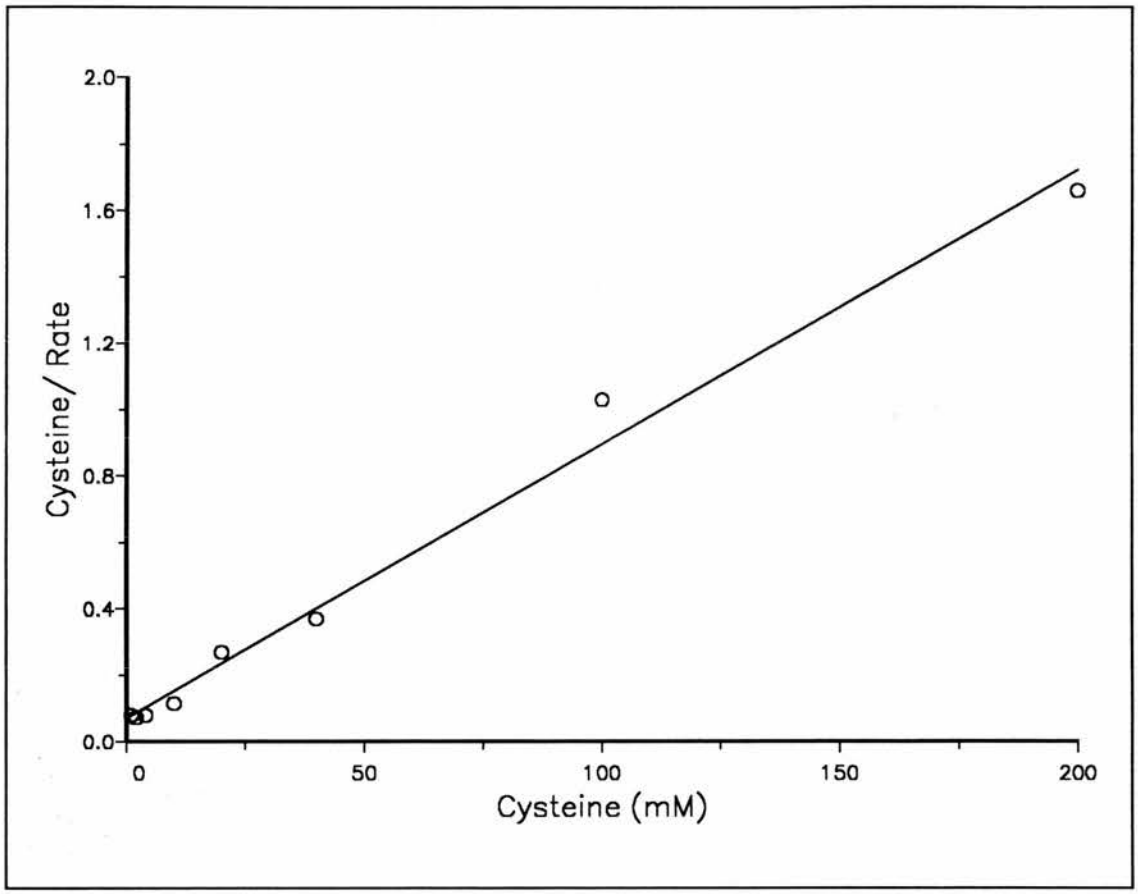


Figure 4.22 Hanes Plot of Cysteine for the Cysteine Dependent NAD'ase. A K_m of 4.4 mM and V_{max} of 300 pmol.min^{-1} was estimated.

The dose response curve obtained for cysteine for the cysteine dependent NAD'ase was fitted to the Michaelis-Menten equation using non-linear least squares curve fitting and the linearised form of the data are shown as the Hanes plot in Figure 4.22. Apparent substrate kinetics for cysteine were observed, and a K_m value of 4.4 mM was calculated.

Separation of the products of the NAD'ase assay on ODS-HYP reverse phase column are shown in Figure 4.23 Separation of ADP-ribose and NAD^+ was achieved

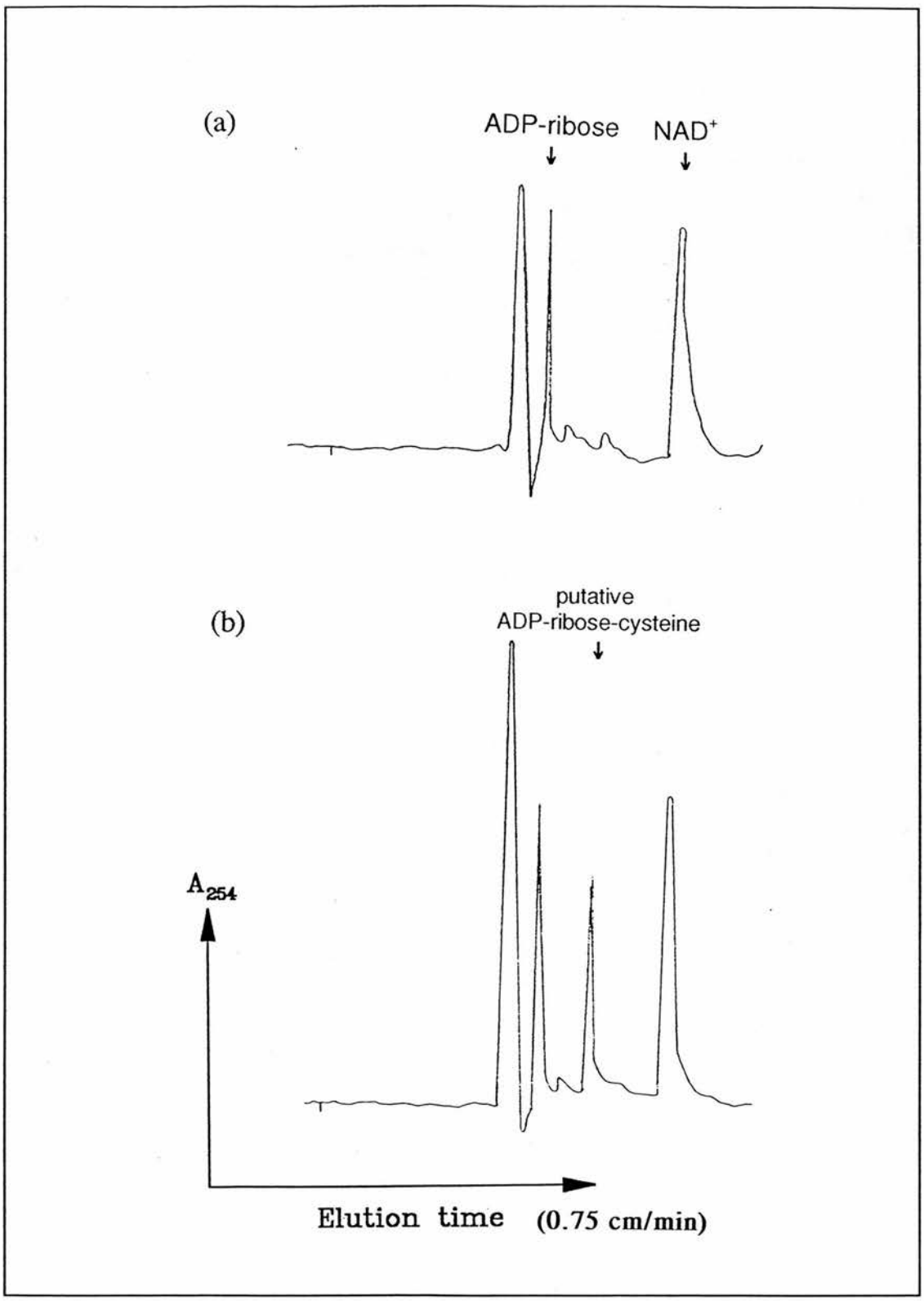


Figure 4.23 Separation of the Products of the Cysteine Dependent NAD'ase Catalysed Reaction on Reverse Phase HPLC. Reactionss contained 100 mM cysteine, 100 μM [^{3}H]NAD$^+$ and were incubated for 15 min at 30°C without (a) and with (b) the enzyme.

by isocratic elution in 10 mM ammonium phosphate pH 3.5, 10 % methanol. Elution times of 4.8 min for ADP-ribose and 7.5 min for NAD^+ were consistent between runs. Incubations which contained cysteine and cysteine dependent NAD'ase showed little change in the size of the ADP-ribose or NAD^+ peaks detected at 254 nm. A second peak was observed at 5.5 mins, and was found to contain ^3H label (Figure 4.24). This is in a similar position to the putatative ADP-ribosylcysteine product described for pertussis toxin by Lobban and van Heyningen. Treatment of this novel peak with phosphodiesterase yielded a new ^3H labeled peak at 16.6 min, coincident with the AMP standard as shown in Figure 4.25.

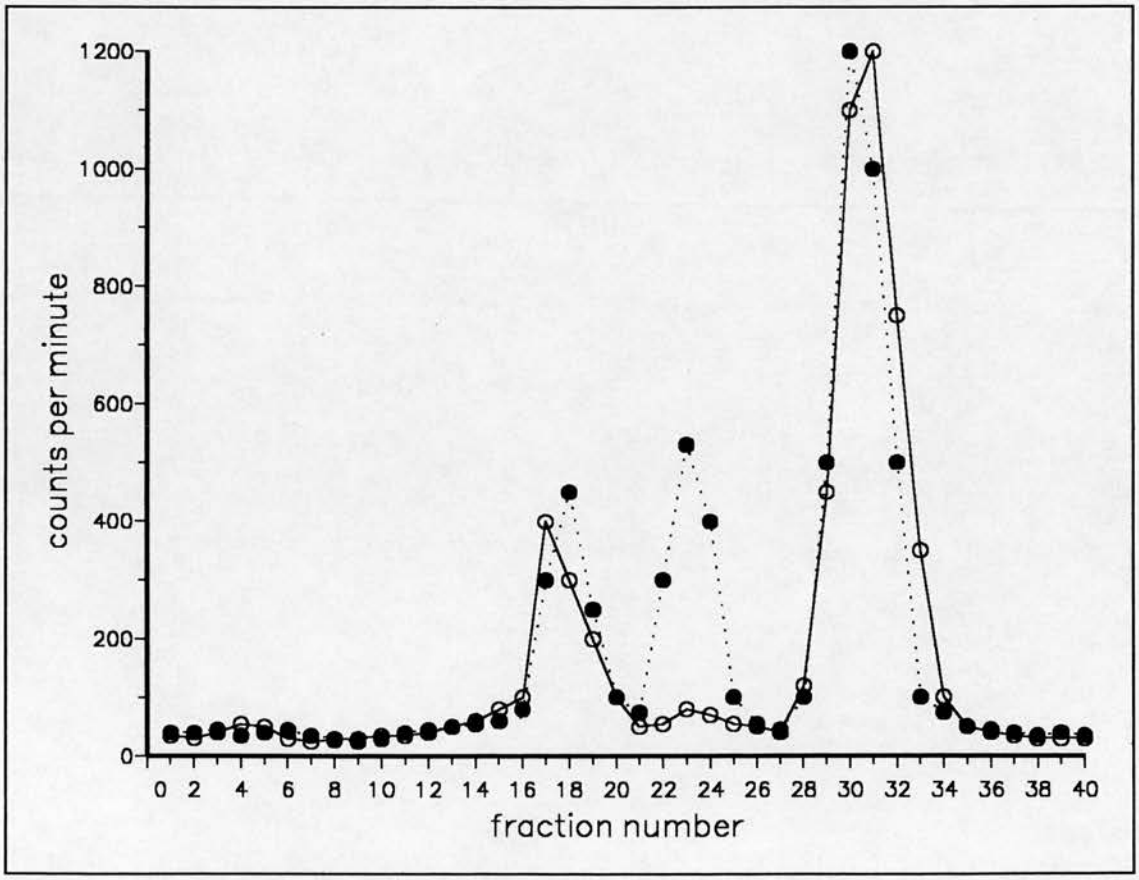


Figure 4.24 Counts Detected in the Eluate from the Separation of the Products of the Cysteine dependent NAD'ase Assay on Reverse Phase HPLC, with (●) and without (○) enzyme added.

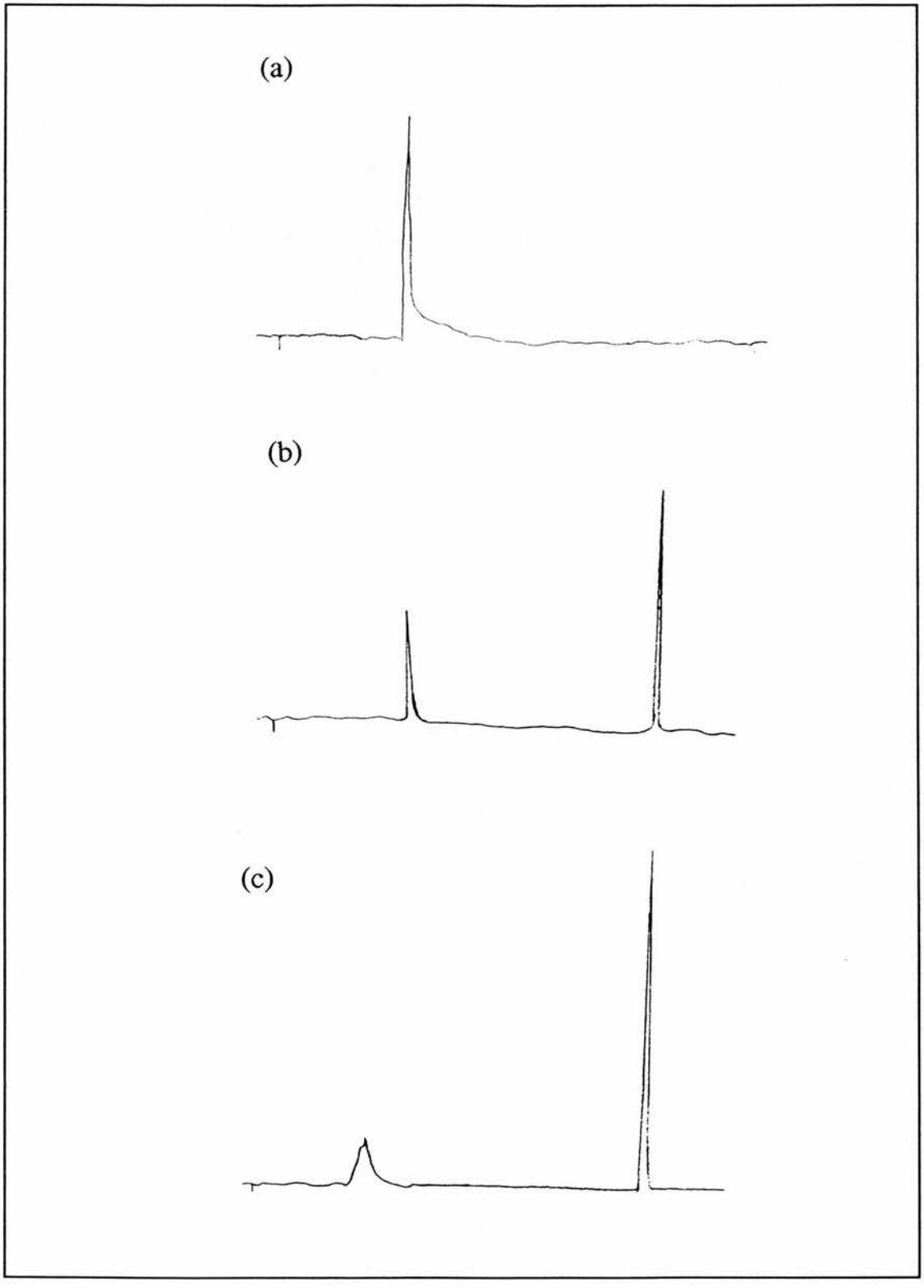


Figure 4.25 Phosphodiesterase Treatment of the Putative ADP-ribosyl-cysteine product. The putative ADP-ribose containing compound (a) was treated with phosphodiesterase and the products of the reaction were separated by reverse phase HPLC (b). The major product released was compared to an AMP standard (c) run under comparable conditions 1 ml/min, 2.67 cm/min.

4.3.5 Substrate Specificity of Cysteine Dependent NAD'ase

Is the reaction amino-acid specific or can any amino-acid act as an acceptor eg arginine? Can cysteine be susbtituted for any thiol containing compound and if so, is cysteine an acceptor or an activator? To answer these questions NAD'ase assays were set up containing saturating levels of NAD^+ (100 μM), 50 mM potassium phosphate buffer, pH 7.4 and 100 ng of cysteine dependent NAD'ase. Nicotinamide released in the presence or absence of potential substrates (100 mM argininemethylester, 100 mM glycine, 100 mM cysteine, 100 mM dithiothreitol and 100 mM S-carboxymethylester cysteine) was then determined.

4.3.5.1 Results

The NAD'ase activity observed in the presence of potential substrates is listed in table 4.3. No activity was observed with arginine methylester, glycine or S-carboxymethylester cysteine. A dose response curve for dithiothreitol shows that like cysteine apparent substrate kinetics were observed. The data were fitted to the Michaelis-Menten equation using non-linear least squares curve fitting and are shown in Figure 4.26 as the Hanes plot. A K_m value of 0.1 mM was calculated. This was 40 fold lower than that observed for cysteine. DTT is a powerful reducing reagent. The difference in apparent K_m between cysteine and DTT may reflect this property. 30% of the maximal activity observed with cysteine was observed in the presence of DTT. Pre-incubation of the cysteine dependent NAD'ase with 10 mM DTT did not effect the apparent substrate kinetics observed for cysteine; a K_m 4 mM was observed.

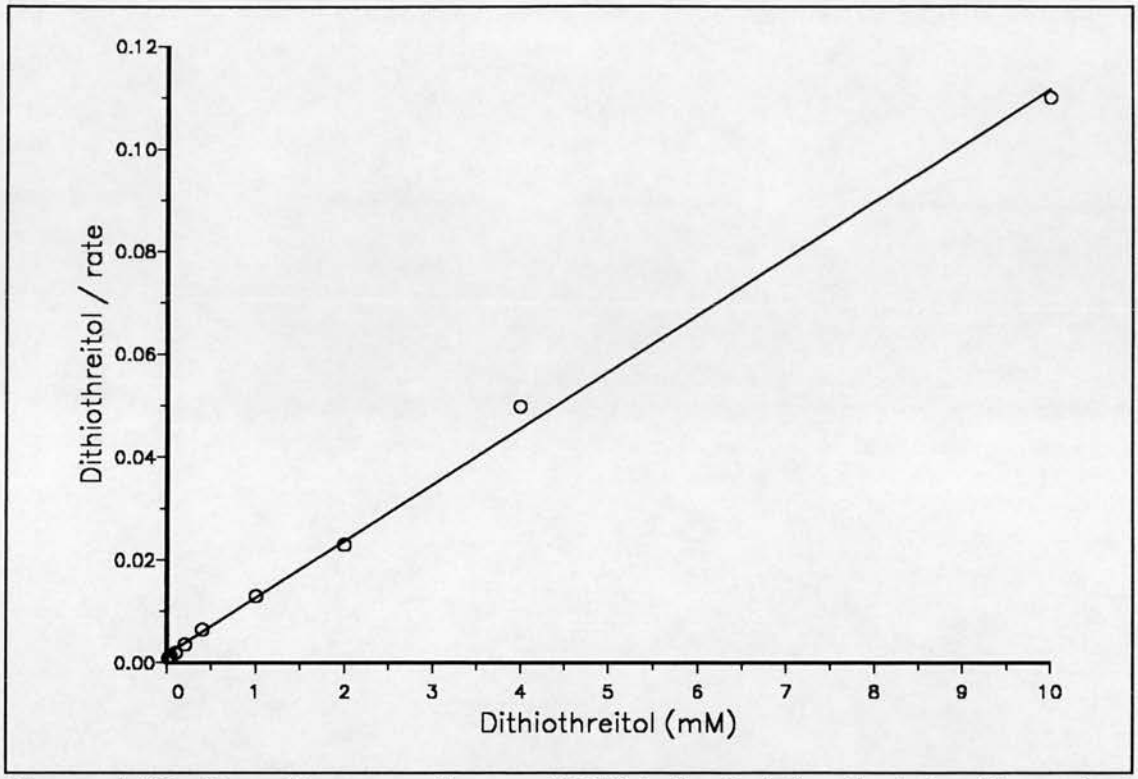


Figure 4.26 Dose Response Curve of Dithiothreitol for Cysteine Dependent NAD'ase. K_m of 0.1 mM and V_{max} of 87 pmol·min^{-1} was estimated.

Table 4.3 Substrate Specificity of Cysteine Dependent NAD'ase

SUBSTRATE	K_m (mM)	V_{max} (pmol/min)
cysteine	4	300
arginine-methylester	> 100	not detectible
glycine	> 100	not detectible
dithiothreitol	0.1	87
S-carboxymethylester-cysteine	> 100	not detectible

4.3.6 Discussion

4.3.6.1 Time Course

Comparison of the time courses shown in Figure 4.1 and Figure 4.17 for pertussis toxin and cysteine dependent NAD'ase respectively shows that 30% of the total NAD^+ is hydrolysed by pertussis toxin in 180 min, compared to 15 min for the cysteine dependent NAD'ase. One hundred fold more protein was required for the pertussis toxin catalysed reaction compared to the mammalian catalysed reaction. The pertussis toxin reaction was linear until 50% of NAD^+ had been hydrolysed, and was stable at 30°C. The cysteine dependent NAD'ase was linear until 30% of the total NAD^+ had been hydrolysed, and was less stable. Incubation of the cysteine dependent NAD'ase for longer periods proved unsuccessful. The rate of nicotinamide release became non-linear after 30 min, even if NAD^+ was still available. The lability of the enzyme observed during the purification (chapter three) is most probably the cause of this problem.

4.3.6.2 pH Optima

The simplest model to explain the pH optima curves observed is that at least two ionizing groups present in the active site are able to change their state of protonation as the pH changes. For catalytic activity to be observed only one of these groups need be protonated. The bell shaped curve observed is steep; 1.5 pH units separate the curves at half the maximal height. Accurate determination of the pK_a of the ionizable groups is not possible from this simple analysis. However, an estimate of the pK_a by measuring the pH at the points where half maximal activity was observed gives pK_a 6.8 for the first ionizable group and 8.1 for the second. It is difficult to attribute specific ionizable groups to these pK_a values, since the pK_a will be dependent upon the environment of the charged group within the active site. For example, the γ-carboxyl group of glutamate has a pK_a of 3.9 as a free amino acid, but has a pK_a 6.5 within the active site of lysozyme[155]. Site directed mutagenesis and photoaffinity cross-linking experiments of pertussis toxin have implicated the importance of Glu-129 and His-35 residues in catalysis. A mechanism has been proposed where Glu-129 stabilizes an oxocarbonium ion intermediate of NAD^+ and His-35 acts as a proton acceptor for the incoming acceptor molecule[156]. Given the

similarity of the pH profiles it is inviting to suggest that the catalytic mechanisms of the two enzymes are similar with the important residues in similar environments within their respective active sites.

4.3.6.3 Substrates of cysteine dependent NAD'ase

NAD^+ had a high affinity for cysteine dependent NAD'ase: a K_m value of 8 μM was determined. This is a similar order of magnitude to the affinity of NAD^+ for pertussis toxin (30 μM) and the NAD^+: arginine ADP-ribosyltransferase from turkey erythrocytes (8 μM)[59]. It is approximately 100 fold tighter binding than that observed for cholera toxin (1.1 mM) and six fold tighter binding than that described for a NAD^+: cysteine ADP-ribosyltransferase from human erythrocytes (65 μM)[75]. From the Vmax observed at saturating NAD^+ and saturating cysteine a k_{cat} may be estimated for the cysteine dependent NAD'ase. Assuming that all the protein added is active and that the enzyme has a molecular weight of 45,000 (estimated from SDS polyacrylamide gels) then the cysteine dependent NAD'ase has a k_{cat} of 30 min^{-1}. This is 150 fold higher than the k_{cat} of 0.2 min^{-1} estimated for pertussis toxin (calculated from the M_r 28,000 of the active subunit S1, rather than the whole toxin, 110,00).

Apparent substrate kinetics were observed for cysteine and a K_m value of 4 mM was calculated. This shows a twenty fold higher specificity for cysteine than pertussis toxin (100 mM), and a similar specificity to that exhibited by the NAD^+: cysteine ADP-ribosyltransferase (4.4 mM). The presence of the free thiol group was essential for activity to be observed; no NAD glycohydrolase activity was observed in the presence of S-carboxymethylester cysteine. Arginine-methylester and glycine were not substrates, confirming that the enzyme is specific for cysteine and is free from any contamination by NAD^+: arginine ADP-ribosyltransferase. NAD glycohydrolase was observed in the presence of DTT and a K_m value of 0.1 mM was calculated. These data sugggest that the role of thiol reagents may be two fold. Free thiol may be required to reduce a disulphide bond in order to observe NAD'ase activity and also act as acceptors for ADP-ribose. This is similar to the properties observed for thiol reagents with respect to pertussis toxin, as discussed in section 4.2.3.

Evidence that cysteine was an acceptor for ADP-ribose was suggested by the identification of a novel ADP-ribose containing product isolated by reverse phase chromatography. This product was eluted between ADP-ribose and NAD^+ in a similar position to that described for ADP-ribosyl-cysteine, eluted under identical conditions by Lobban and van Heyningen[145]. The product was only observed in incubations containing cysteine, NAD^+ and cysteine dependent NAD'ase. From these observations the presence of cysteine in this product was assumed. The possibility that this product was derived from the non-enzymatic reaction of ADP-ribose and cysteine as described by McDonald *et al.*[147] remains. However, much shorter incubation times were used to investigate the activity of the cysteine dependent NAD'ase; 30 min instead of overnight incubation of the reactants with pertussis toxin. This time period may be too short for the non-enzymatic chemistry to play a significant role. Also, no product was observed in the absence of cysteine dependent NAD'ase, despite the presence of significant levels of ADP-ribose (20 μM) from the hydrolysis of NAD^+ stock solution with storage. It seems more likely that the product observed was generated enzymatically from these observations.

ADP-RIBOSYLTRANSFERASE ACTIVITY

5.1 Introduction

The work presented in this chapter investigates the ability of the purified cysteine dependent NAD'ase from bovine erythrocyte to also catalyse the transfer of ADP-ribose to a protein target. As discussed more fully in chapter one this reaction was first identified during the study of the pathogenesis of diseases such as diphtheria, cholera and whooping cough. Many of the bacterial toxins specifically modify members of one family of proteins, the G proteins, which are important in signal transduction pathways. Endogenous ADP-ribosyltransferase activities which apparently modify the same target proteins have been identified, as well as a variety of novel target proteins suggesting a ubiquitous role for mono-ADP-ribosylation in cellular control mechanisms.

Mono-ADP-ribosylation catalysed by the bacterial protein toxins is amino acid specific. Diphtheria toxin modifies diphthamide residue of elongation factor 2 thereby disrupting protein synthesis. Cholera toxin modifies an arginine residue of $G_s\alpha$ and pertussis toxin modifies a cysteine residue of $G_i\alpha$, thus in both cases disrupting the cAMP dependent signalling pathway. At high concentrations cholera toxin has been shown to modify a variety of proteins *in vitro* eg BSA[157], acetyl CoA carboxylase[158] and by auto-ADP-ribosylation, itself[159]. In these reactions ADP-ribose is always covalently linked through an arginine residue, but are not thought to be physiologically relevant. Pertussis toxin on the other hand is more specific, ADP-ribosylating only the α subunit of trimeric G proteins which contain a C-terminal cysteine residue (G_i, G_o, and transducin).

Endogenous ADP-ribosylation of arginine, cysteine, diphthamide, lysine, asparagine and glutamate residues of various target proteins have been reported (Chapter one for review). Few of the enzymes responsible have been identified, but those that have appear to be amino acid specific, eg NAD^+: arginine ADP-ribosyl transferase from turkey erythrocytes and NAD^+: cysteine ADP-ribosyltransferase from

human erythrocytes. It is possible therefore, that for every potential ADP-ribosylated target there is a specific ADP-ribosyltransferase to be discovered.

Endogenous enzymes need not be present to account for all the ADP-ribosylated protein identified. Free ADP-ribose is a highly reactive species and may react with proteins directly by the formation of Schiffs base with lysine residues[160,161]. Arginine residues in skeletal muscle cytosolic proteins have been shown to be specifically modified by free ADP-ribose[162]. The glycolytic enzymes glyceraldehyde-phosphate dehydrogenase, lactate dehydrogenase and alcohol dehydrogenase have all been shown to be covalently modified through a cysteine residues by free ADP-ribose[114] or NAD^{+}[115] leading to inhibition of the enzymes.

Care must be taken therefore in interpreting apparent ADP-ribosyltransferase activity. Ideally the target protein and the modified residue can be identified by protein sequencing. Experimentally this may be difficult because the target protein is present in such low quantities. The type of ADP-ribose-amino acid linkage can be deduced from the stability of the radiolabel to chemical cleavage. Jacobson and co-workers[163] have worked out conditions to release ADP-ribose selectively from all the known ADP-ribose-amino acid linkages and these are summarised in table 5.1.

The aim of the purification procedure, described in chapter three, was to purify a "pertussis toxin like" endogenous ADP-ribosyltransferase, ie an enzyme which could catalyse the transfer of ADP-ribose from NAD^{+} to cysteine residue of G_i. This hypothesis was tested using erythrocyte ghost membranes. Erythrocyte ghosts are a well characterised membrane system[164] and have been used to study ADP-ribosylation by bacterial toxins[165] and endogenous enzymes[76, 77]. Three quarters of the proteins present in the erythrocyte membrane comprise of structural proteins eg Spectrin, tektrin, myosin-like and actin-like polypeptides. Minor constituents which have been identified include glyceraldehyde-3-phosphate dehydrogenase, protein kinase, adenylate cyclase and Na^{+}/K^{+} ATP ase.

The ADP-ribosylation of bovine erythrocyte membrane proteins catalysed by pertussis toxin, cholera toxin and the purified cysteine dependent NAD'ase were compared. Labelling of G proteins was confirmed by recognition of ADP-ribosylated protein with anti-bodies raised against the C-terminal tails of G_i and G_s. Then, ADP-

Table 5.1 The Stability of the Amino acid -ADP-ribose Linkage

LINKAGE	FORMIC ACID (44%)	SODIUM HYDROXIDE (1M)	WEAK ALKALI (pH 9.0)	HYDROXYL AMINE (pH 7.0)	MERCURIC ION (10 mM)
ARGININE	stable	released	stable	released	stable
ASPARAGINE	stable	stable	stable	stable	stable
CYSTEINE	stable	released	stable	stable	released
DIPHTHAMIDE (HISTIDINE)	stable	stable	stable	stable	stable
LYSINE (KETOAMINES)	stable	released	released	stable	stable
SERINE OR THREONINE	released	stable	stable	stable	stable

ribosylation of a cysteine residue on the target protein was tested in two ways. The first involved pretreatment of the membrane proteins with N-ethylmaleimide (NEM), a sulphydryl alkylating reagent which should block the available ADP-ribosylation sites of the target protein. The second tested the sensitivity of the labelled protein to mercuric ion. The thio glycosidic bond formed between cysteine and ADP-ribose is sensitive to nucleophilic attack by the mercuric ion[166]. It was confirmed that ADP-ribose was released by separation of the products by ion exchange chromatography.

Finally the sensitivity of the endogenous enzyme to known inhibitors of ADP-ribosyltransferases and NAD'ases was tested. Thymidine was originally described as a specific inhibitor of poly(ADP-ribose) polymerase[167], however it also inhibits mono-ADP-ribosyltransferase activity. A concentration of 1 mM causes 60 % inhibion of NAD^+: arginine ADP-ribosyltransferase activity from turkey erythrocytes[59]. Isoniazid is a specific inhibitor of NAD'ases and was originally designed as an anti-tuberculosis drug[168]. Different vertebrate species have different sensitivities. Sensitive species

include birds and ruminants, K_i about 0.1 mM. Non sensitive species include rodents, horses and humans. These reagents are commonly used in ADP-ribosylation incubations containing crude membrane preparations to minimise the loss of [^{32}P]NAD$^+$ [169].

5.2 Methods

5.2.1 ADP-ribosylation of Membrane Proteins

5.2.1.1 Preparation of Bovine Erythrocyte Ghosts

Washed bovine erythrocytes were prepared as described in chapter three, section 3.2.1. The cells were lysed with hypotonic buffer 10 mM potassium phosphate, 0.1 mM PMSF, 0.01% (v/v) 2-mercaptoethanol, 1 mM benzamidine HCl pH 7.8. The ghosts and unbroken cells were collected by centrifugation (14,000 g, 25 min) and were washed with hypotonic buffer three times until no more haemoglobin was detectable in the supernatant. The ghost membranes were incubated with hypotonic buffer containing 0.1 mM EDTA (inverting buffer) for 15 min at 30 °C to invert them (trapped haemoglobin is visibly released). The amount of protein present was estimated using the Lowry assay (section 2.5.1) and the membranes were stored frozen at -70 °C.

5.2.1.2 Preparation of Rat Hypothalamus Membranes

The method of Nestler *et al.*[170] was followed. The hypothalamus was dissected out from the brains of three rats and stored in oxygenated Krebs media, 126 mM NaCl, 5 mM KCl, 4.25 mM NaH_2PO_4, 25 mM $NaHCO_3$, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM D-glucose pH 7.4. The tisssue was homogenised in ice cold homogenising buffer, 50 mM Tris HCl, 6 mM $MgCl_2$, 1 mM EDTA, 3 mM benzamidine, 1 mM DTT, 0.1 mM PMSF pH 8.0 and 5% sucrose. 200 μL homogenising buffer per 1 mg weight weight of tissue was used. The membranes were collected by centrifugation (10000g, 10 min, 4 °C) and resuspended in the homogenising buffer. The amount of protein was estimated using the Lowry assay and the membranes were stored at -70 °C until required.

5.2.1.3 ADP-ribosylation Incubation

The optimised ADP-ribosylation conditions characterised for pertussis toxin by Ribeiro-Neto *et al.*[171] were followed. A typical ADP-ribosylation reaction contained; 20-40 μg bovine erythrocyte membrane proteins, 5 mM ATP, 0.1 mM GTP, 0.1 mM PMSF, 2 mM EDTA, 50 mM sodium phosphate pH 7.0 and 0-10 μg activated pertussis toxin (see section 2.2) or 0 - 200 ng purified cysteine dependent NAD'ase in a reaction volume 45 μL. A control assay reaction was performed containing all of the above ingredients except pertussis toxin or endogenous enzyme. The reaction was started by the addition of 2 μM [adenylate-^{32}P]NAD^+ (20-30 mCi/mmole) and incubated at 30°C for 30 min. The reaction was stopped by the addition of 10% ice cold tri-chloroacetic acid (TCA) and the proteins were allowed to precipitate on ice for 10 min. The membrane proteins were collected by centrifugation (13000 g, 5 min) and were washed three times with 0.1% TCA to remove excess NAD^+.

ADP-ribosylation of bovine erythrocyte ghosts by 5 μg cholera toxin was achieved using a modification of the above procedure. 10 mM $MgCl_2$ was added to promote the activation of G_s α, the substrate for cholera toxin. Magnesium is not included in pertussis toxin incubations because it inhibits ADP-ribosylation of G_i α[172,45].

ADP-ribosylation of rat hypothalamus membranes required a further modification of the assay conditions because the membrane preparation was very crude. The ADP-ribosylation conditions of Gill and Coburn[170], which were designed to minimise NAD^+ depleting reactions, were followed. Typicaly, incubations contained all of the components described above for the erythrocyte assay plus 10 mM thymidine, 10 mM isoniazid, 10 mM creatine kinase and 5 units/mL creatine phosphokinase. Thymidine and isoniazid inhibit poly(ADP-ribose) synthetase and NAD'ases which will deplete the NAD^+ available to the toxin. Creatine phosphate and creatine phoshokinase are present as an ATP re-generating system.

5.2.1.4 Detection of ADP-ribosylated Proteins

After precipitation and washing, the membrane proteins were solubilised in 2% (w/v) SDS, 50 mM Tris-HCl pH 6.8 as described in section 2.5.2.2. The proteins were separated on a 10% SDS polyacrylamide gel and were stained with Coomassie blue. The gel was dried and then exposed to Hyperfilm-MP (Amersham) using Kodak screen intensifiers for 24-48 h at -70°C. The autoradiograph film was developed using an automatic X-ray film developer.

5.2.1.5 Quantification of the Amount of ADP-ribosylated Protein

The amount of label per lane was quantified by scanning the density of the band using a scanning densitometer (Joyce/loebl). To account for any uneven protein loading, the density of erythrocyte band V (45 kDa) visualised by coomassie staining was measured. A relative measure of the amount of labelling was determined from:-

$$\frac{\textit{peak area of ADP-ribosylated product}}{\textit{peak area of erythrocyte band V}}$$

The peak area was calculated for at least two different time exposures and only samples from the same gel exposed under identical conditions were compared. It was therefore not necessary to take the precaution of pre-exposing the film to ensure a linear response over the time course of the exposure.

5.2.2 Cross Reactivity with G_i and G_s Antibodies

A Western blot analysis of the ADP-ribosylated proteins was performed as described in section 2.5.3. Erythrocyte ghost proteins separated on 10% SDS polyacrylamide gel were transferred onto nitro-cellulose (1 A, 2 h). The transfer of pre-stained markers (Biorad) was used to assess the effeciency of transfer. Excess binding sites were blocked with 1% dried milk solids, 0.1 % TWEEN in Tris buffered saline (TBS). Antibodies which had been raised in rabbits, against the C-terminal decapeptide of $G_s\alpha$ and $G_i\alpha$[173] were used in 1/2000 dilution to probe the blot. Anti-rabbit horseradish peroxidase IgG conjugate was used as the second antibody in a 1/4000 dilution. The anti-body labelling was detected by ECL.

5.2.3 Identification of the Amino Acid Linkage

5.2.3.1 NEM Treatment of Membranes

N-ethyl-maleimide (NEM) is a powerful sulphydryl alkylating reagent and will readily react with reactive thiol groups on proteins to form an acid resistant adduct. It is important, first, to remove any free thiol present in the preparation, and second, to use fresh NEM. NEM is hydrolysed at pH 7.0 and above to form inactive N-ethylmaleamate.

Prepared bovine erythrocyte membranes were washed three times with 50 mM phosphate buffer pH 7.2, 0.1 mM PMSF, 0.1 mM EDTA to remove excess 2-mercaptoethanol. 1 mM NEM (freshly prepared) was then added to the membranes and incubated at 30°C, for 30 min. NEM was removed by washing the membranes three times with inverting buffer. A control membrane preparation was treated as above, except NEM was not added. ADP-ribosylation of these treated membranes was performed as described in section 5.2.1.3.

5.2.3.2 Release of Label

The sensitivity of the ADP-ribosyl-protein linkage to cleavage by neutral hydroxylamine or mercuric ion was tested. After precipitation and washing of the membrane proteins the sample was divided into three equal portions. One was treated with 0.5 M neutral hydroxylamine, 0.5 M NaCl pH 7.4 at 30°C for a maximum of 3 h. Another was treated with 10 mM mercuric acetate, pH 3.0 at 30°C for a maximum of 30 min. The last was set up as a control incubation. The sample was incubated in 50 mM potassium phosphate buffer, 0.5 M NaCl, pH 7.4 for a maximum of 3 h. The precipitate was not solubilised under these conditions, but remained as a fine white precipitate in suspension. The precipitate was collected by centrifugation (13,000 g, 5 min) and the proteins were then solubilised and separated out on 10% SDS polyacrylamide gels as described in section 5.2.1.4. Pertussis toxin catalysed ADP-ribosylation of rat hypothalamus membranes were used as a standard for ADP-ribose-cysteine-protein.

5.2.3.3 Detection of Released Label

To detect the type of label released by mercuric acetate it was necessary to remove excess NAD^+ efficiently. This was achieved by first separating the products on 10% SDS polacrylamide gels, and then electro-eluting the labelled protein (3 W, constant power, 3 h) as described in section 2.5.4. The eluted protein was then treated with 10 mM mercuric acetate, at 30°C, for 30 min. The protein was precipitated by adding 5% (v/v) perchloric acid. The supernatant was neutralised by addition of potassium hydroxide (4°C) and the insoluble salt (potassium perchloride) formed was removed by centrifugation (13,000 g, 5 min). The sample was diluted two fold with 100 mM potassium phosphate pH 4.5 before applying to a Spherisorb S5-SAX HPLC column (see section 2.4.2), which had been equilibrated in 100 mM potassium phosphate buffer pH 4.5. The products were separated on the column by isocratic elution. 0.25 mL fractions were collected and 4 mL scintillant (Pharmacia, HiSafe) added. The samples were counted for 10 min using a Packard 1900CA scintillation counter.

5.3 Results

5.3.1 ADP-Ribosylation of Erythrocyte Ghost Membrane Proteins

The labelling observed in erythrocyte ghost membranes after incubation with [^{32}P-adenylate]NAD^+ and pertussis toxin, cholera toxin, purified cysteine dependent NAD'ase or membranes only are shown in Figure 5.1. The membrane proteins were separated out on a 10% SDS polyacrylamide gel and lane (a) shows the proteins visible after staining with Coomassie blue. The major polypeptides of the red blood cell membrane identified by Steck[165] are indicated. After exposure of the separated proteins to autordiography film for 48 h, several radiolabelled bands were detectible (lanes (b) to (e)). The molecular weight of these labelled polypeptides was estimated by comparison with the relative mobility of known molecular weight standards, as indicated. Free [^{32}P]NAD^+ ran with the dye front during electrophoresis and was washed out of the gel during the protein staining procedure, such that only low levels of radioactivity was detected in the dye front. Some of this activity may have been due to radiolabelled low molecular weight proteolytic fragments.

The major radiolabelled product observed was a 55 kDa band. The labelling in this region decreased in the presence of cholera toxin but was increased relative to the control in incubations containing cysteine dependent NAD'ase and pertussis toxin. This band is not coincident with any of the major polypeptides visible with Coomassie staining, suggesting that a minor component of the erythrocyte membrane has been labelled. The pattern of radiolabelled products for incubations containing pertussis toxin and cysteine dependent NAD'ase were similar; two bands of high molecular weight at the top of the gel, a 60 kDa band, enhanced labelling of a 55 kDa band and very faint labelling of a 29 kDa protein. However, only incubations with pertussis toxin showed labelling of a 41 kDa band. Cholera toxin produced unique labelling of a 66, 50, 45 and 42 kDa polypeptides. The appearance of the high molecular weight doublet, 66 kDa band and 29 kDa band were variable between membrane preparations, whereas the labelling of the 55 kDa protein was consistent.

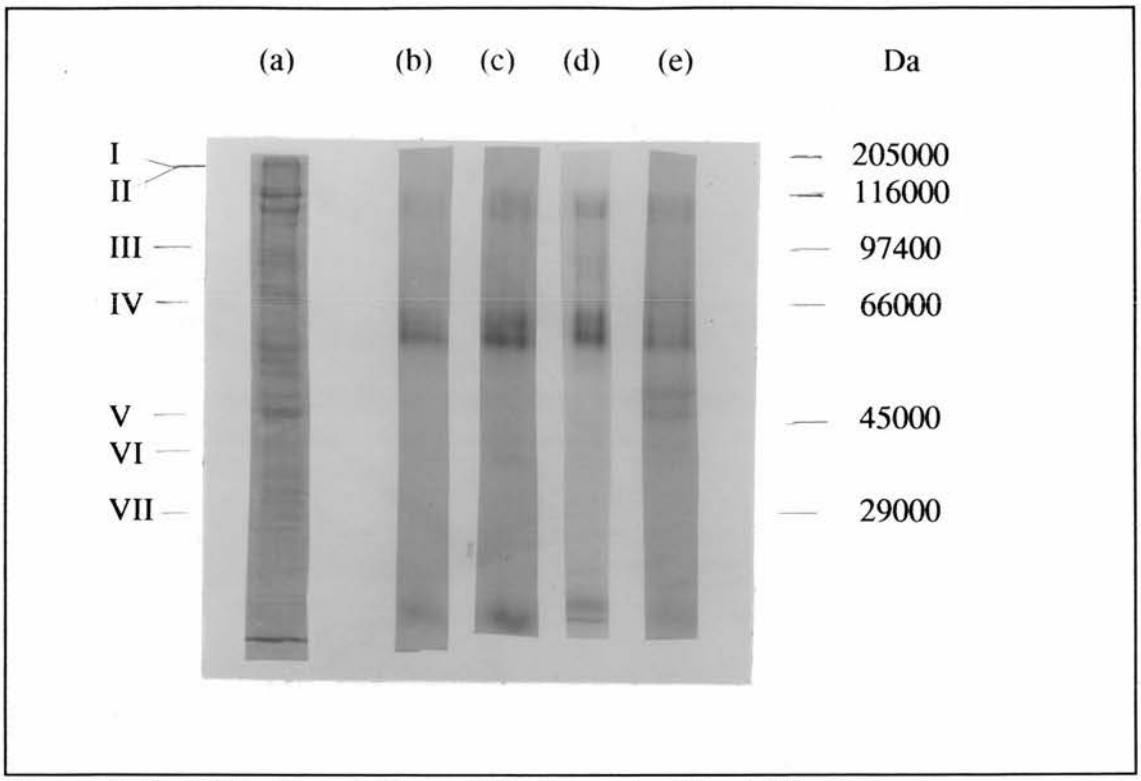


Figure 5.1 ADP-ribosylation of Erythrocyte Ghost Membranes
Lane (a) Coomassie staining of erythrocyte ghost proteins with the major erythrocyte ghost proteins I - VII indicated. The autoradiograph of ADP-ribosylated ghost proteins after incubating the membranes with [^{32}P]NAD^{+} and membranes only (b), cysteine dependent NAD'ase (c), pertussis toxin (d) and cholera toxin (e) are shown.

5.3.1.1 Dose Response

The effect of adding increasing amounts of cysteine dependent NAD'ase or pertussis toxin to the ADP-ribosylation assay was investigated. Figure 5.2 shows the autoradiographs of the 50-60 kDa region for incubations carried out for 15, 30 and 60 min, containing 0 - 200 ng of purified cysteine dependent NAD'ase. The density of labelling was quantified (as described in section 5.2.1.5) and are shown in the graph. Each incubation contained 15 μg of membrane proteins and the separated proteins were exposed to the autoradiograph film for 36 hours. Very low levels of labelling was observed after 15 min. After 60 min incubations a dose response on additionof cysteine dependent NAD'ase was difficult to detect because background labelling was also high and amount of target protein becomes limiting. A dose response was observed, following a 30 min incubation, most clearly. A maximum three fold increase in the amount of labelling of the 55 kDa protein was observed on

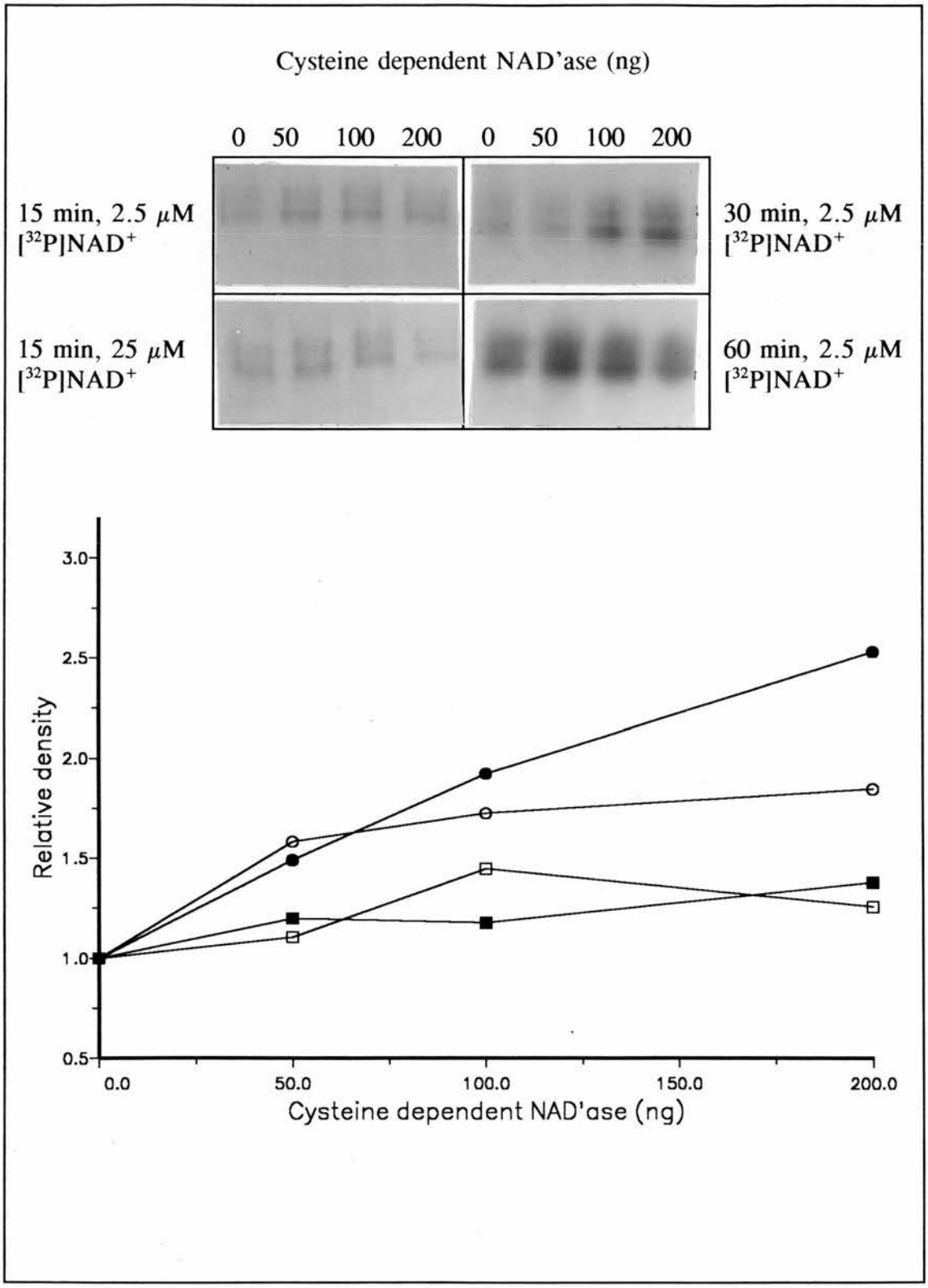


Figure 5.2 Labelling of Erythrocyte Ghost Membrane Proteins: Dose response for the Cysteine Dependent NAD'ase. 15μg of erythrocyte ghost membranes were incubated with 0-200 ng cysteine dependent NAD'ase and 2.5 μM [^{32}P]NAD$^+$ for 15 (○), 30 (●), and 60 minutes (■) at 30°C. The 15 minute incubation was repeated with 25 μM [^{32}P]NAD$^+$ (□).

addition of 100 ng of cysteine dependent NAD'ase. Further additions of NAD'ase did not increase the amount of labelling; in fact a reduction was observed.

Long incubation times and high concentrations of cysteine dependent NAD'ase in the assay do not produce high levels of labelling. This may be explained by the fact that the NAD'ase activity of the enzyme hydrolyses all the available NAD^+ during the time course of the assay or only a limited amount of target protein is available for modification. To test whether the maximal labelling observed was due to substrate depletion duplicate assays were set up. One contained 2.5 μM NAD^+ and the other 25 μM NAD^+ (specific activity of both was 20 mCi/mmole). The cysteine dependent NAD'ase dose response was repeated at both concentrations. No further increase in labelling in the presence of a high concentration of NAD^+ was observed, suggesting that NAD^+ was not a limiting factor for the enzymatic reaction.

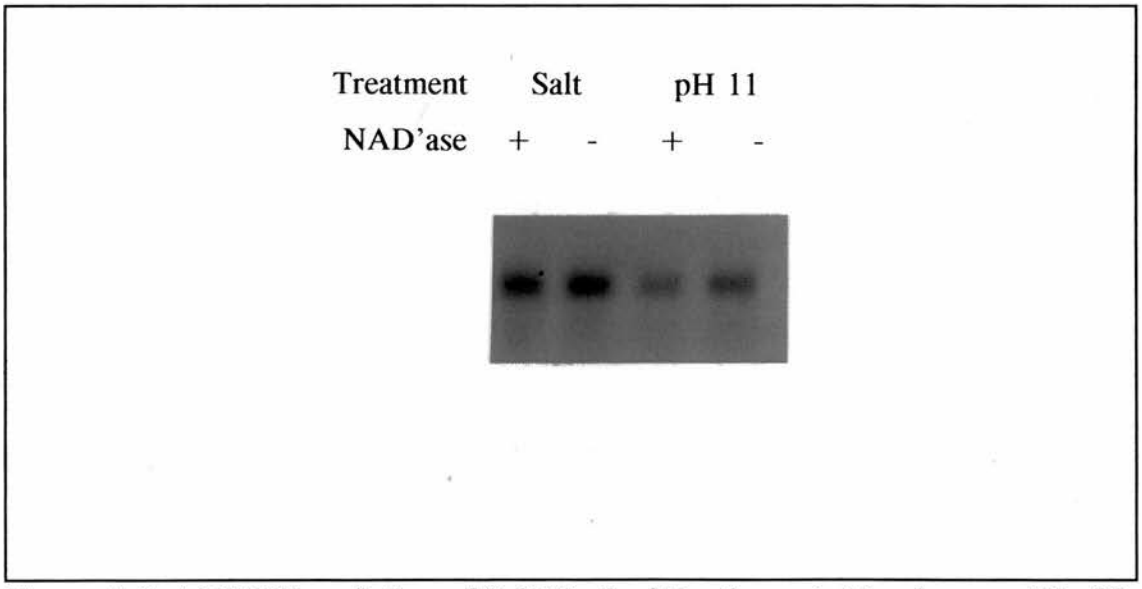


Figure 5.3 ADP-Ribosylation of Salt Washed Erythrocyte Membranes. The 80-35 kDa region of the autoradiograph is shown.

Unexpectedly high levels of endogenous ADP-ribosyltransferase activity, in the incubations containing erythrocyte ghost membranes only, was observed. To determine whether this activity was membrane associated or due to contamination by cytosolic proteins, the membranes were washed using more stringent conditions. The ghost membranes were treated with 50 mM potassium phosphate, 200 mM NaCl

pH 7.2 or 0.1 M potassium carbonate buffer pH 11.0. Under these washing conditions only integral membrane proteins should remain; cytosolic and loosely associated membrane proteins should be stripped away. The ADP-ribosylation experiments were repeated after these washing procedures and labelling of a 55 kDa protein was observed again in the presence or absence of added ADP-ribosyltransferase (Figure 5.3). The washing procedures reduced the level of the background labelling, but did not inhibit it completely, suggesting that the background activity must be membrane associated. ADP-ribosylation of the 60 kDa band was no longer observed after these more stringent washing conditions. This suggests that the 60 kDa protein was loosely associated with the membrane, whereas the 55 kDa protein must be an integral membrane protein which was not removed by washing with salt or high pH.

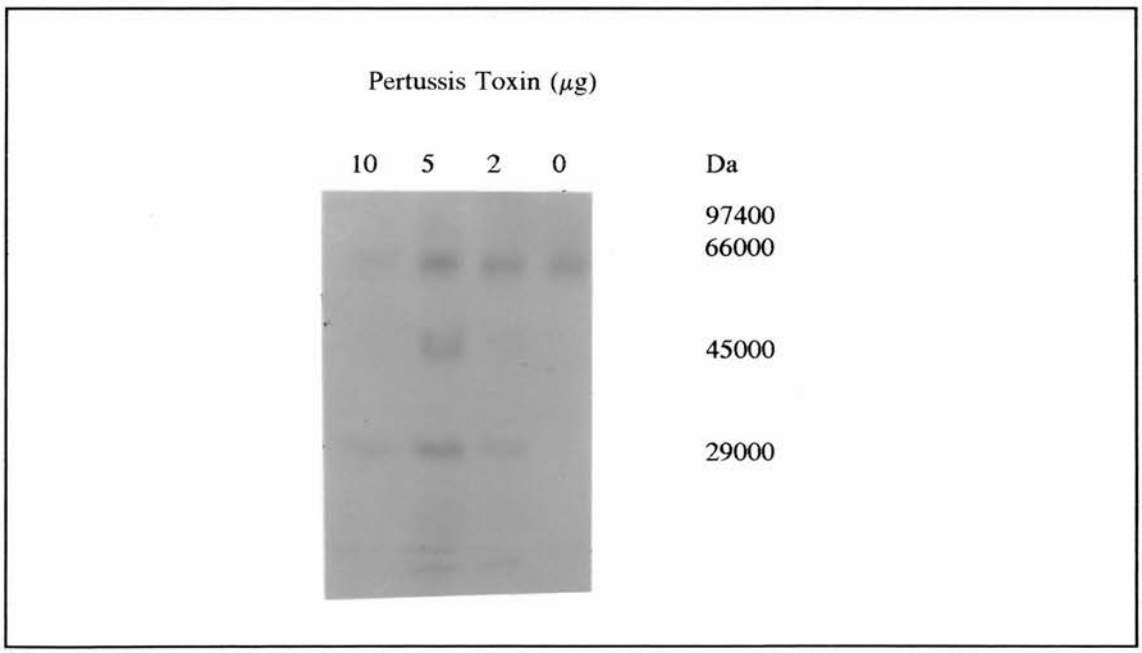


Figure 5.4 Labelling of Erythrocyte Ghost Membrane Proteins: Dose Response of Pertussis Toxin

Figure 5.4 shows the labelling observed after incubating erythrocyte ghost membranes with pertussis toxin (0 - 10 μg) and [^{32}P]NAD^{+} for 30 min. Addition of 5 μg of activated pertussis toxin increased the amount of labelling of the 55 kDa band two fold. Labelling of 41kDa and 27 kDa was maximal for 5 μg. Further additions of toxin lead to a reduction in the amount of labelled protein observed. The NAD'ase

activity of the pertussis toxin may become significant at high concentrations of toxin in the assay. The toxin may have hydrolysed the NAD^+ substrate before significant levels of target proteins were modified.

5.3.1.2 Reaction of Free ADP-ribose with Membrane Proteins

The possibility that the labelling of erythrocyte membrane proteins observed was due to the non-enzymatic reaction of susceptible groups on the protein with ADP-ribose was investigated. The incubation conditions used were a modification of those of Ribeiro-Neto *et al.*[171] in which inhibitors of NAD'ase and poly(ADP-ribose) were not included. Erythrocytes ghosts have been reported to be good systems for ADP-ribosylation as they have low levels of NAD^+ depleting reactions. Erythrocyte membranes are known to have a NAD'ase on the outer surface of the cell[174]. In the ADP-ribosylation assay inverted re-sealed ghosts were prepared such that the membrane associated NAD'ase activity should be shielded from the reactants. However carryover of low levels of NAD'ase could generate a significant amount of ADP-ribose, which may modify proteins directly and account for the background labelling observed. The increased levels of labelling observed on addition of the cysteine dependent NAD'ase or pertussis toxin may result from the increased production of ADP-ribose.

The NAD'ase activity of the cysteine dependent NAD'ase used in the assay was determined. A rate of 5 pmol min^{-1} in the presence of 10 μM NAD^+ and 10 mM cysteine was found. If this rate were to occur during the ADP-ribosylation reaction then 68% of the total NAD^+ would be used up. However, the ADP-ribosylation conditions are such that NAD^+ and reducing reagent are present at sub-saturating levels, so cysteine dependent NAD'ase activity will be minimised.

Free [adenylate-^{32}P]ADP-ribose was prepared from [^{32}P-adenylate]NAD^+. 10 μM [^{32}P]NAD^+ (20 mCi/mmole) was incubated with 10 μg pig brain NAD'ase (15 nmol.min^{-1}. mg^{-1}) (Sigma, E.C. 3.2.2.5.) in 50 mM potassium phosphate buffer, pH 7.2 at 30°C for 30 min. The protein was removed by filtration through a

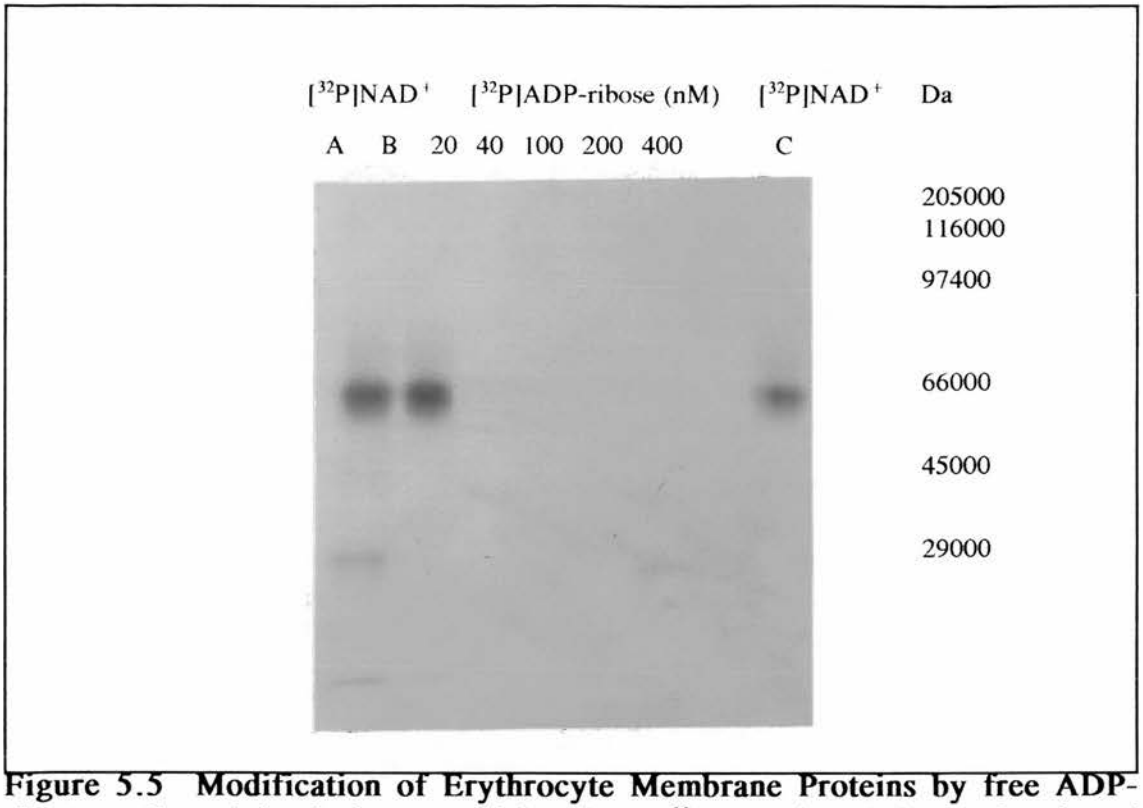


Figure 5.5 Modification of Erythrocyte Membrane Proteins by free ADP-ribose. Control incubations containing 1μM [^{32}P]NAD$^+$ and 5 μg of activated pertussis toxin (A), 100 ng cysteine dependent NAD'ase (B) or membranes only (C).

membrane, which had a M_r cut-off of 10,000. The filtrate was applied to S5-SAX column and ADP-ribose was separated from NAD$^+$ as described in materials and methods section 2.4.2. ADP-ribosylation reactions were set up containing 40 μg membrane proteins and a range of ADP-ribose concentrations (0 - 0.4μM) equivalent to a maximum of 45% NAD$^+$ hydrolysed. Control incubations containing 1 μM NAD$^+$ with and without cysteine dependent NAD'ase or pertussis toxin were set up. Figure 5.5 conclusively shows that the ADP-ribosylated 55 kDa band observed in control and experimental samples is not due to reaction of free ADP-ribose liberated during the time course with susceptible groups on the membrane protein.

5.3.2 Cross Reactivity G_i and G_s Antibodies

The ADP-ribosylation of a 55 kDa protein by the purified enzyme and pertussis toxin was observed. This is too high a molecular weight for G_i (39 - 42 kDa), although it is within the range for G_s (42 - 55 kDa). However, it is not uncommon for proteins to run anomalously on SDS polyacrylamide gel electrophoresis. The acid precipitation and prolonged solubilisation in 0.1% SDS buffer at 80°C may have caused cross linking of proteins to occur or the ADP-ribosylation modification itself may have altered the mobility of the protein. To test these possibilities and to confirm endogenous ADP-ribosylation of G protein the cross reactivity of the labelled protein with G_i and G_s antibodies was investigated.

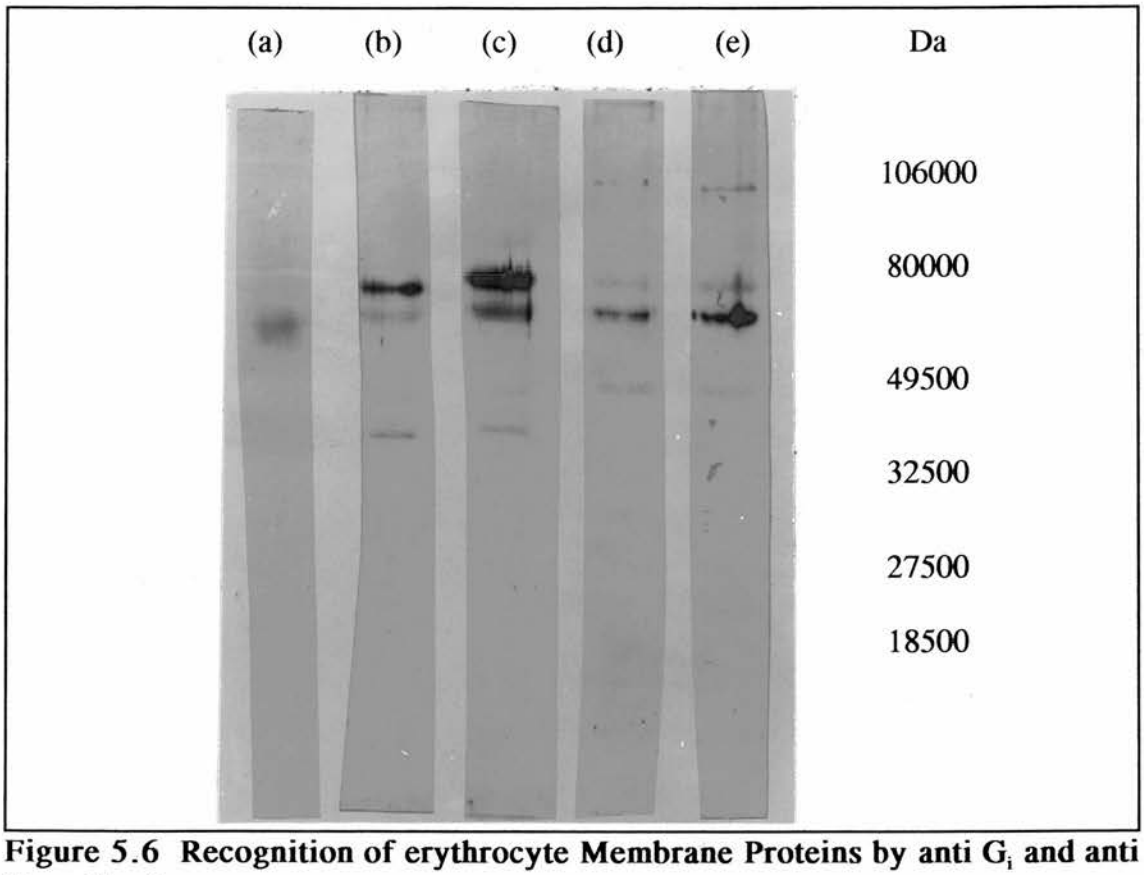


Figure 5.6 Recognition of erythrocyte Membrane Proteins by anti G_i and anti G_s antibodies.

Lane (a) shows the autoradiograph of erythrocyte ghost proteins labelled by the cysteine dependent NAD'ase. Western blots of untreated and ADP-ribosylated membranes probed with anti-peptide antibodies raised against $G_i\alpha$ are shown in lanes (b) and (c), respectively. Lanes (d) and (e) show the Western blots of untreated and ADP-ribosylated membranes probed with anti-$G_s\alpha$ anti-peptide antibody. The antibodies were detected using ECL.

A Western blot analysis of ADP-ribosylated erythrocyte ghost proteins and corresponding autoradiograph are shown in Figure 5.6. A maximum of 10 min exposure time was required for detection of the antibody labelling. Exposure of the radiolabelled protein to film for this time did not produce any detectible bands. After five hours the ECL reagent was used up and further exposure of the blot (in the absence of radiolabel) did not produce any detectible bands on the film. The blot was then exposed to Hyperfilm MP for 24 h to detect the radiolabelled protein. Experiments were repeated in which the radiolabelled proteins were detected before treatment with the anti-bodies. No difference in the pattern of ADP-ribosylated protein or antibody labelling was observed. All the bands observed result from interaction of the second antibody (anti-rabbit-HRP conjugate) with the anti-peptide antibody. Incubation of the membranes with second antibody only, or ECL reagents only, did not produce any detectible bands.

The reaction of anti G_i and anti G_s α antibodies with treated and untreated membranes are shown in Figure 5.6. The relative intensities of the bands recognised by the antibodies were unchanged and no shift in the bands recognised by the antibodies was observed between the labelled and unlabelled proteins. These data suggest that the ADP-ribosylation conditions do not inhibit the recognition of G_i and G_s by the anti- peptide antibody and do not cause a shift in the mobility of G_i and G_s on SDS-PAGE. Overlay of the blot and autoradiograph images showed that the 55 kDa ADP-ribosylated band was not recognised by the C-terminal anti-peptide antibodies of $G_i\alpha$ or $G_s\alpha$. This is seen more clearly by examination of the scanning densitometry data shown in Figure 5.7. $G_i\alpha$ anti-peptide antibody recognises 41 kDa (faint labelling), 71 kDa and 64.5 kDa (heavy labelling) proteins. $G_s\alpha$ anti-peptide antibody recognises a 48 kDa and 64,500 kDa protein. The antibodies are known to cross react with BSA and this may account for the labelling observed at 64 - 71 kDa. The radiolabelled band is broad and partially overlaps with the 65 KDa band recognised by both antibodies. However, the most intensely radiolabelled portion of the band is not recognised by either antibody, suggesting that modification of a novel acceptor protein has occurred.

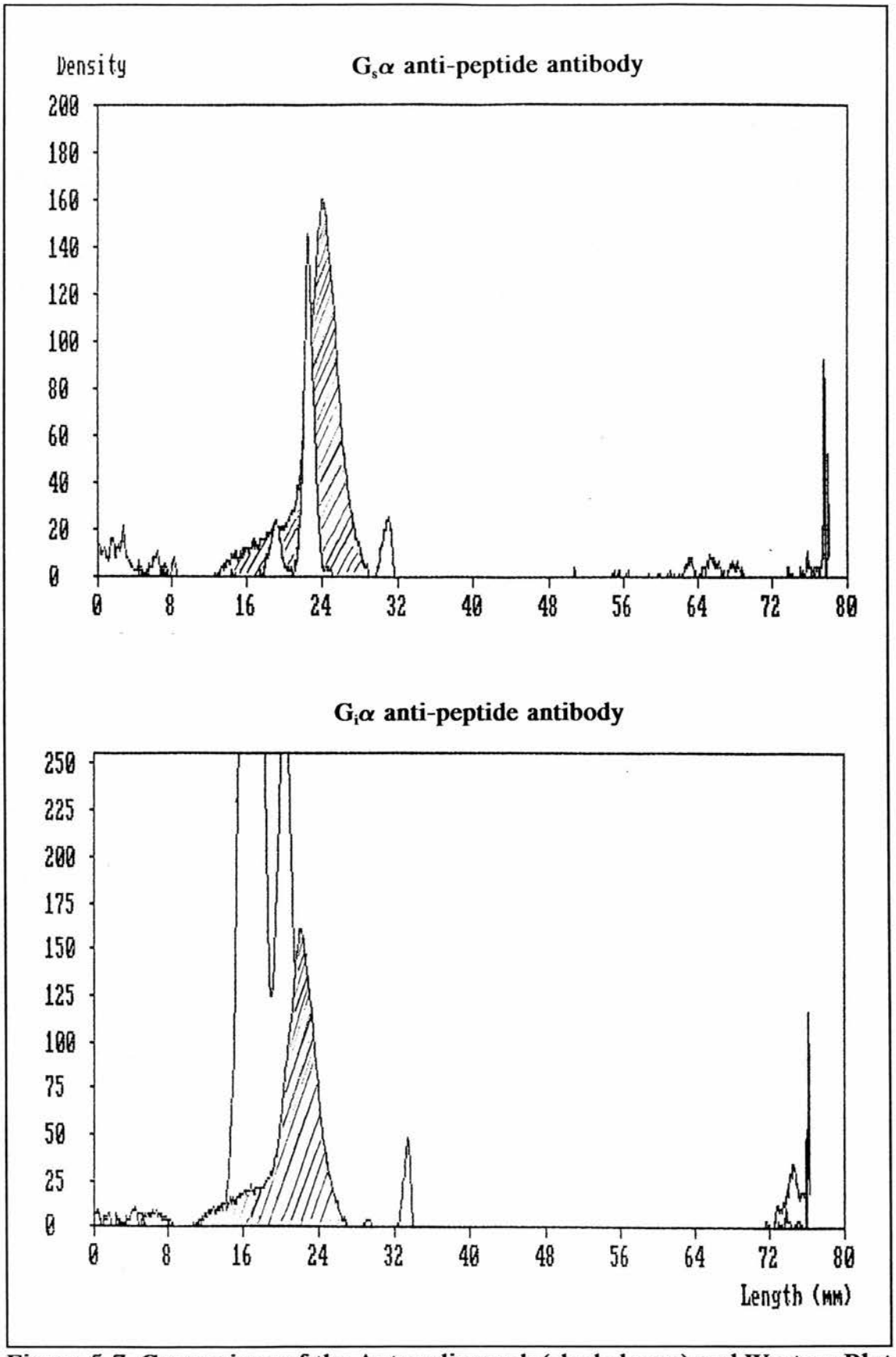


Figure 5.7 Comparison of the Autoradiograph (shaded area) and Western Blot of ADP-ribosylated Erythrocyte Ghost Proteins by Scanning Densitometry.

5.3.3 Identification of the type of ADP-Ribosyl Amino Acid Linkage

5.3.3.1 Sensitivity of ADP-ribosylation Observed to Pre-Treatment with NEM

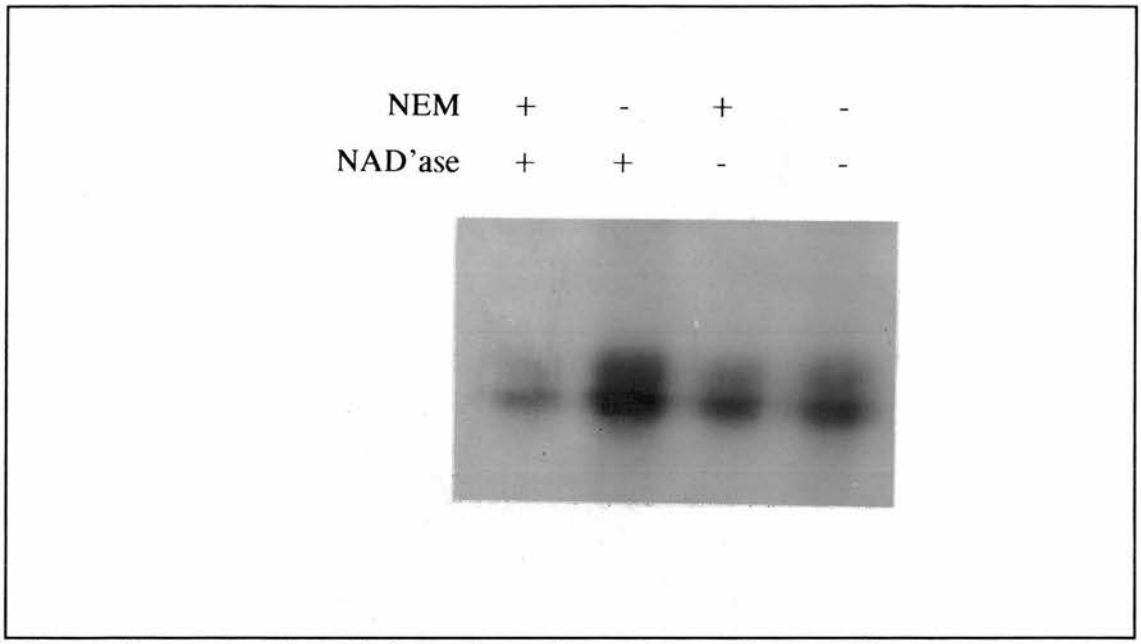


Figure 5.8 ADP-ribosylation of Erythrocyte Membranes after Pre-Treatment with NEM

Free thiol groups associated with the erythrocyte membrane proteins were alkylated using NEM. The ability of the cysteine dependent NAD'ase to ADP-ribosylate these modified proteins is shown in Figure 5.8. The background membrane associated ADP-ribosylation was not effected, whereas the enhanced labelling of the 55 kDa protein by the cysteine dependent NAD'ase was inhibited by NEM treatment of the membranes. This result is consistent for a modification of a cysteine residue on the 55 kDa protein and suggests that the background labelling may result from the labelling of a different residue, which is insensitive to treatment with NEM, on the same protein or a different protein with a similar molecular weight.

5.3.3.2 The Sensitivity of ADP-Ribosyl-Protein to Neutral Hydroxylamine and Mercuric Acetate

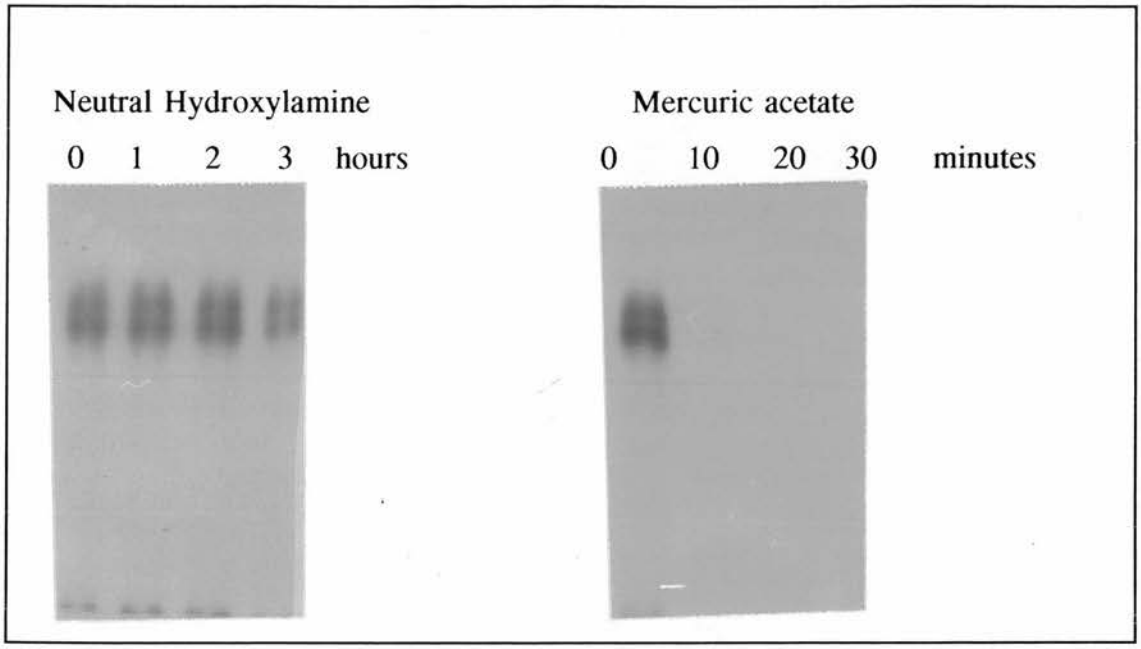


Figure 5.9 The Sensitivity of ADP-Ribosyl-G_i to Hydroxylamine and Mercuric Ion

The conditions required to cleave ADP-ribosyl-cysteine were tested using ADP-ribosylated G_i prepared from hypothalamus membranes incubated with pertussis toxin. Hypothalamus membranes were chosen because they were a better source of pertussis toxin substrate and background membrane associated activity was very low. Approximately 100 fold more G protein was detected with $G_i\alpha$ anti-peptide antibody in the hypothalamus membranes compared to the erythrocyte membranes. Figure 5.9 shows the time dependent release of radiolabel in the presence of 10 mM mercuric acetate or 0.5 M hydroxylamine pH 7.4, 0.5 M NaCl. The release of [^{32}P]ADP-ribose was rapid in the presence of mercuric ion; no label was detectible after 10 mins, at 30°C. No label release was detected with neutral hydroxylamine, even after three hours, thus confirming the stability of the ADP-ribosyl linkage to this nucleophile.

Erythrocyte ghost membranes that had been ADP-ribosylated by endogenous membrane proteins, cysteine dependent NAD'ase or pertussis toxin were treated using

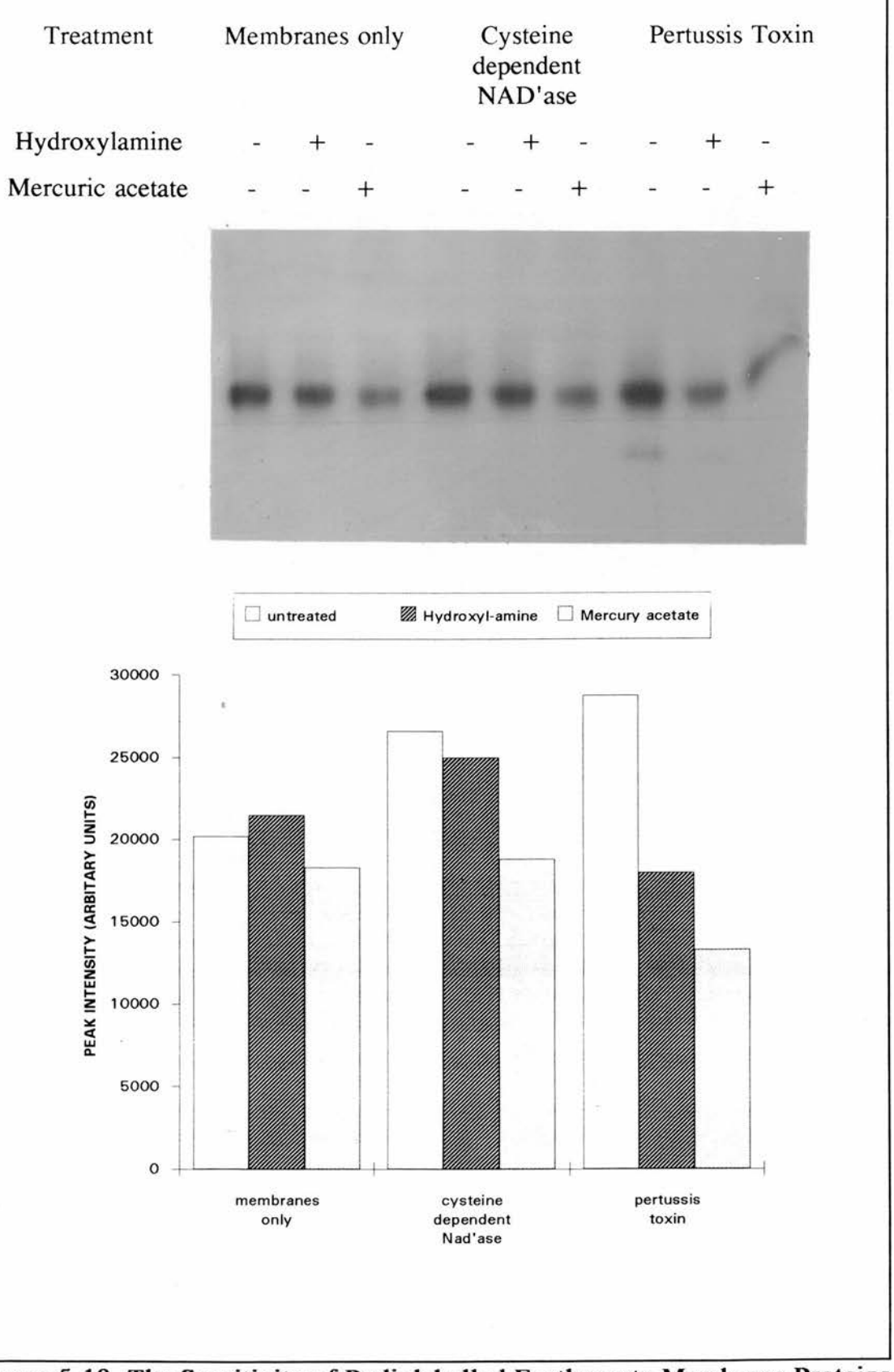


Figure 5.10 The Sensitivity of Radiolabelled Erythrocyte Membrane Proteins to Neutral Hydroxylamine and Mercuric Acetate

the conditions determined for pertussis toxin labelled G_i. The results are shown in Figure 5.10. The amount of label per band was quantified by scanning the intensity of the band using a scanning densitometer and are shown in the graph. Background labelling was apparently stable to both neutral hydroxyl amine and mercury acetate. The increased labelling observed of the 55 kDa protein by cysteine dependent NAD'ase and pertussis toxin was released by mercury acetate, but was stable to hydroxylamine. Pertussis toxin catalysed labelling of a 41 kDa protein was released completely by treatment with mercury acetate.

5.3.3.3 Detection of the Released Label

To confirm that ADP-ribose and not NAD^+ or some other [^{32}P-adenylate]-containing compound was released by mercuric ion the products of the reaction were separated on S5-SAX ion exchange column. The sample was treated as describred in section 5.2.3.3. The elution times of NAD^+ and ADP-ribose that had been treated with mercuric acetate under the conditions of the experiment and untreated fresh NAD^+ and ADP-ribose were compared. The effect of mercuric acetate on NAD^+ during the time course of the reaction was tested to ensure that break down did not occur or the elution time change.

The standards are shown in Figure 5.11 and are detected at 254 nm. Mercuric acetate does not promote the breakdown of NAD^+, and did not effect the elution time of 4.4 min. The elution time of ADP-ribose was effected by the presence of mercuric acetate, changeing from 17 min to 13 min. This change in elution time may be explained by a small change in the pH caused by the treatment with mercury acetate on application of the sample to the column. To account for changes in elution time between samples the position of radiolabelled product detected by scintillation counting was compared to free ADP-ribose and NAD^+ standards run under comparable conditions.

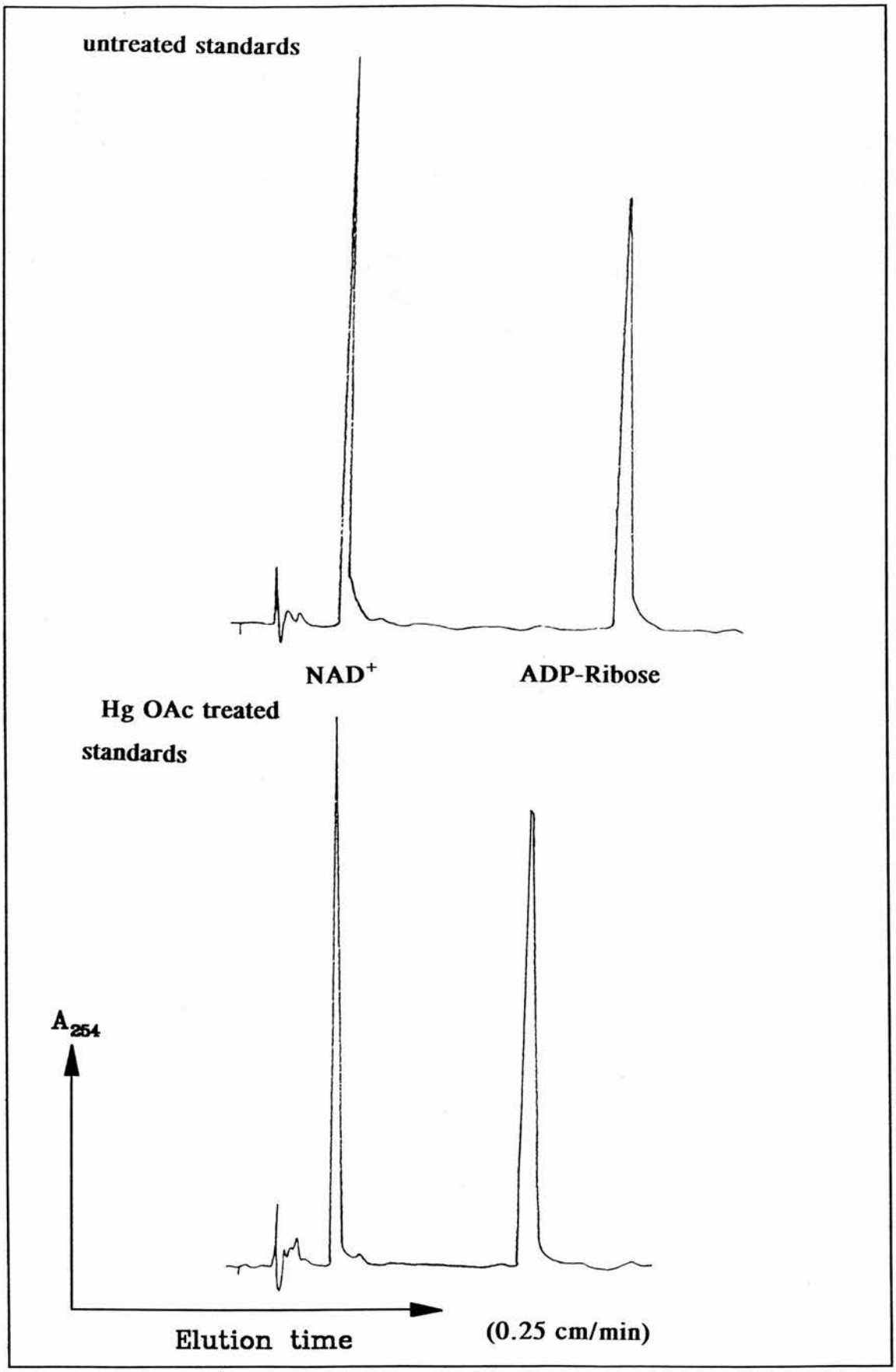


Figure 5.11 NAD^+ and ADP-Ribose Standards: Separation on S5-SAX HPLC

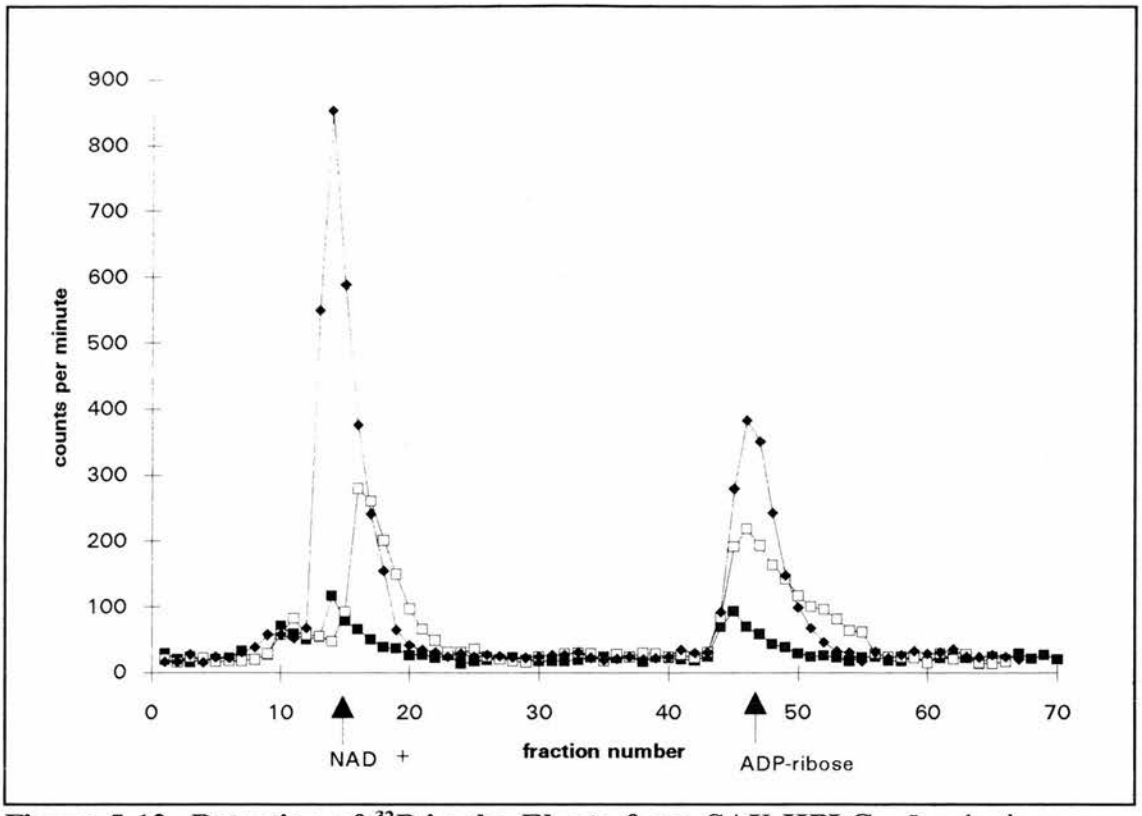


Figure 5.12 Detection of ^{32}P in the Eluate from SAX-HPLC. Incubations were performed containing membranes only (♦), pertussis toxin (□), or cysteine dependent NAD'ase (■).

The radiolabel detected in the column eluate for enzymatic and non-enzymatic ADP-ribosylation incubations are shown in Figure 5.12. Radiolabel was detected in fractions which were coincident with both NAD^+ and ADP-ribose. Incubations containing pertussis toxin and cysteine dependent NAD'ase showed proportionately more ADP-ribose than incubations containing membranes only. The ratio of counts associated with NAD^+ and counts associated with ADP-ribose for pertussis toxin and cysteine dependent NAD'ase containing assays were similar, 0.9 and 2 respectively. Membrane only incubations resulted in five fold less ADP-ribose released relative to NAD^+ (ratio NAD^+: ADP-ribose = 10).

Although the protein precipitate had been washed three times with 0.1 % TCA to remove excess NAD^+ the possibility that some NAD^+ remained trapped within the precipitate cannot be ruled out. To overcome this problem the radiolabelled proteins were separated on a 10% SDS polyacrylamide gel. Only covalently bound radiolabel

will remain with the protein and free NAD^+ and ADP-ribose will run with the dye front. The radiolabelled protein was excised from the gel and the protein electro-eluted as described in section 2.5.4.

The electro-elution conditions used eluted more than 95% of the ADP-ribosylated 55 kDa protein from the gel. No counts were detectible in the gel after elution; 1100 cpm were eluted from the incubations containing membranes only and 1800 cpm from incubations containing cysteine dependent NAD'ase. The electro-eluted protein was treated with mercuric acetate and the protein removed by precipitation with 10% perchloric acid as described in section 5.2.3.3. 100 μM NAD^+ and ADP-ribose standards were added to the sample before application to the column to act as internal controls to indicate any discrepancies in the elution times between runs. The graph in Figure 5.13 shows the counts detected per 0.25 mL fraction collected and the elution times of NAD^+ and ADP-ribose standards detected at 254 nm are indicated. Release of ^3H label by treatment with mercuric acetate was only

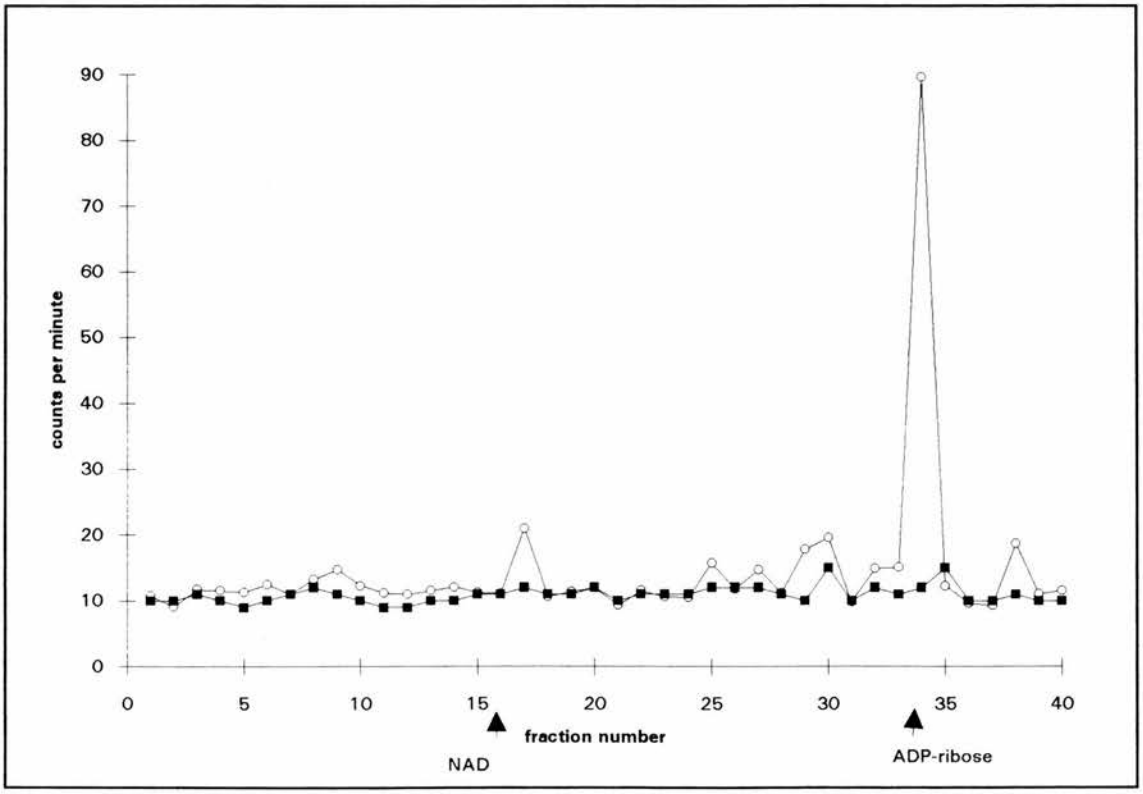


Figure 5.13 Release of Radio-label by Mercuric Acetate Treatment of Electro-eluted 55 kDa Protein which had been labelled in the presence (○) and absence (■) of cysteine dependent NAD'ase.

collected and the elution times of NAD^+ and ADP-ribose standards detected at 254 nm are indicated. Release of ^3H label by treatment with mercuric acetate was only detected for samples which had been incubated with the cysteine dependent NAD'ase. No ^3H counts were released from samples incubated with membranes only. The counts released were low and were equivalent to 5% of the total label incorporated into the 55 kDa band. The electro-elution buffer contained 1% SDS and this may have inhibited the reaction of the mercuric ion with the ADP-ribose-protein substrate. The released ^3H counts were clearly coincident with ADP-ribose. These results suggest that a mono-ADP-ribosyl-cysteine on the 55 kDa had been formed and rule out the possibility of the labelling being due to covalent modification of the protein by NAD^+.

5.3.4 Thymidine and Isoniazid Inhibition

The inhibitory properties of these two commonly used inhibitors of NAD^+ depleting reactions in ADP-ribosyltransferase incubations were tested. Incubations containing inhibitor with and without cysteine dependent NAD'ase were set up. Figure 5.14 shows the autoradiograph of the 55 kDa region and a graph of the relative peak intensity of each lane determined by scanning densitometry. The amount of labelling observed in the presence or absence of the cysteine dependent NAD'ase was compared to the labelling observed from incubations containing membranes only. In the absence of isoniazid and thymidine, a 50% increase in the amount of label incorporated into the 55 kDa band was observed in incubations which contained cysteine dependent NAD'ase. Isoniazid inhibited both the background and cysteine dependent NAD'ase catalysed labelling, such that no significant difference in the amount of labelling between the control and the experiment could be measured. Thymidine inhibited both background and cysteine dependent NAD'ase catalysed ADP-ribosylation. 50% inhibition was observed compared to untreated membranes and no significant difference between control and experiment could be detected.

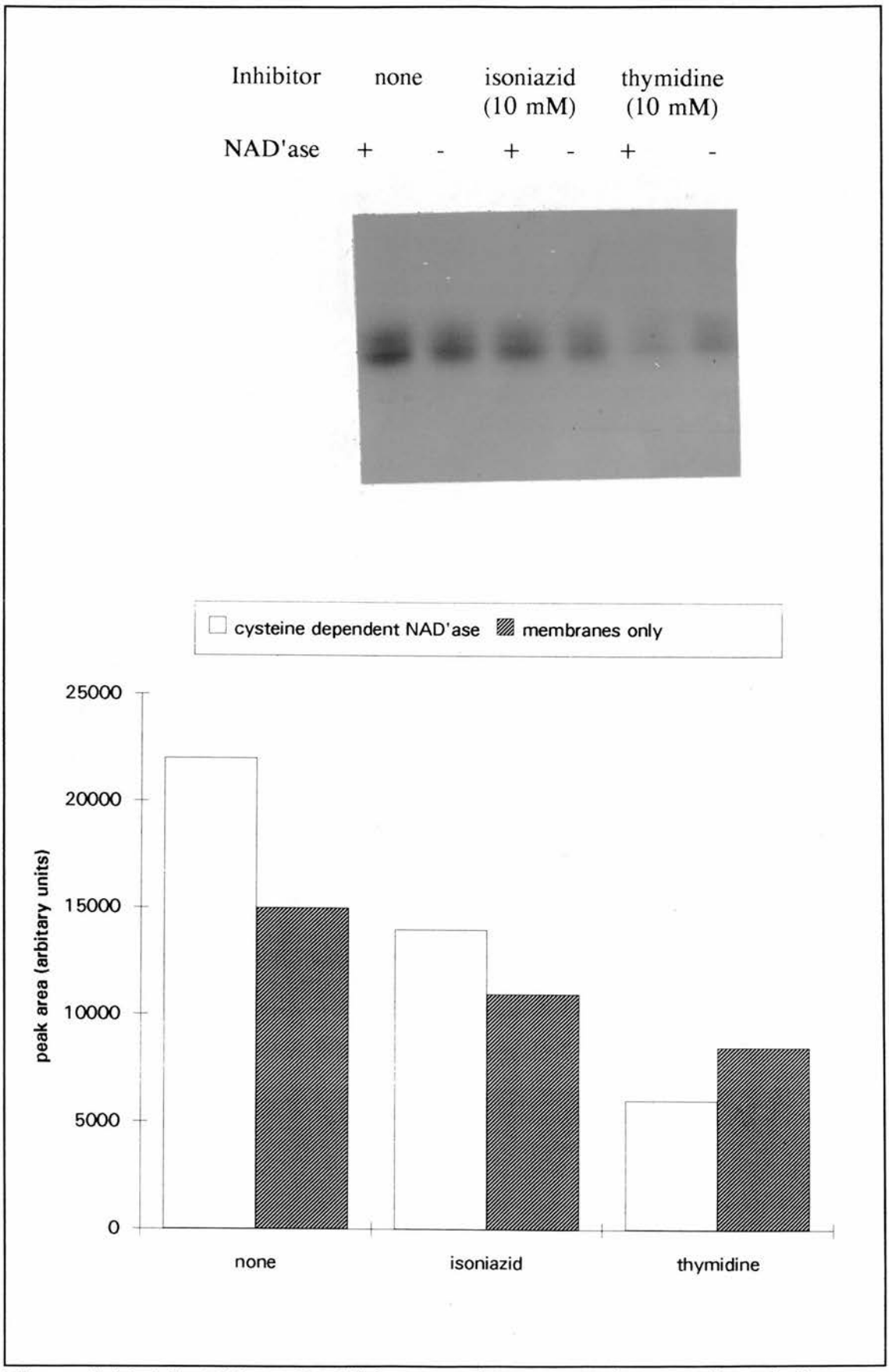


Figure 5.14 The Effect of Inhibitors on the Extent of ADP-Ribosylation

5.3.4.1 Inhibition of NAD glycohydrolase

The inhibition properties of these compounds were investigated further using the NAD glycohydrolase assay as a model system. The NAD'ase assay was performed at saturating (100 μM) and sub-saturating (33 and 10 μM) concentrations of NAD^+, 50 mM potassium phosphate, 100 mM cysteine pH 7.4. 0 - 10 mM concentrations of inhibitor was added to the assay. The rate of nicotinamide released was calculated and the concentration at which half maximal activity was observed was estimated. More detailed inhibitor kinetic studies were not carried out because there was not enough active enzyme available.

The inhibition curves for isoniazid and thymidine are shown in Figure 5.15 and Figure 5.16. Isoniazid and thymidine inhibited the cysteine dependent NAD'ase assay, and 50% inhibition was observed at 0.9mM and 1.2 mM, respectively, at saturating concentrations of NAD^+. The concentration of isoniazid required to give 50% inhibition was dependent on the concentration of NAD^+ in the assay; 50% inhibition was observed at 0.8 mM in the presence of 33 μM and 0.6 mM in the presence of 10 μM NAD^+. These data suggest that isoniazid was a competitive inhibitor against NAD^+ of the cysteine dependent NAD'ase activity. The inhibition observed in the presence of thymidine was independent of the concentration of NAD^+ in the assay; 50% inhibition was observed at 1.2 mM at saturating and sub-saturating concentrations of NAD^+. These data suggest that the inhibition of the cysteine dependent NAD'ase by thymidine was non-competitive with respect to NAD^+.

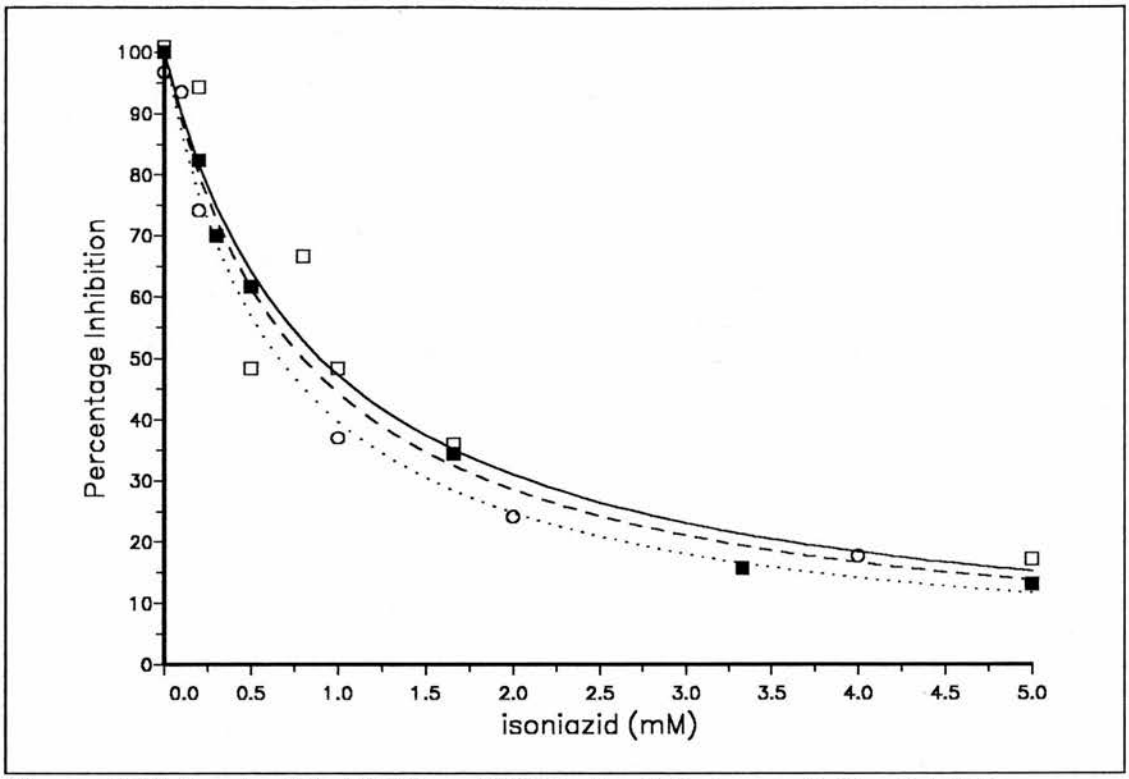


Figure 5.15 Isoniazid inhibition of NAD glycohdrolase activity. Inhibition curves at 100 μM NAD^+(□), 33 μM NAD^+(■) and 10 μM NAD^+(○) are shown.

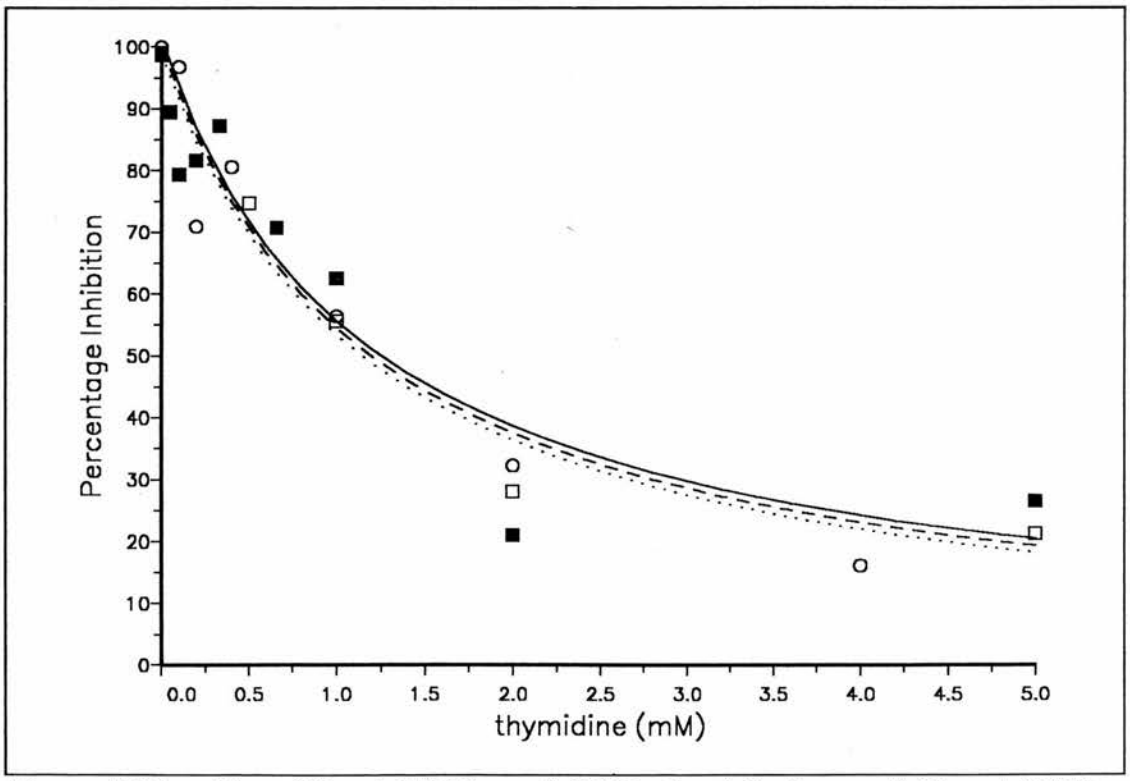


Figure 5.16 Thymidine inhibition of NAD glycohdrolase activity. Inhibition curves at 100 μM NAD^+(□), 33 μM NAD^+(■) and 10 μM NAD^+(○) are shown.

5.4 Discussion

5.4.1 ADP-Ribosylation of Bovine Erythrocyte Ghosts

In this chapter evidence was presented that the cysteine dependent NAD'ase purified from bovine erythrocytes can catalyse the transfer of ADP-ribose from NAD^+ to a target protein found in the bovine erythrocyte. Incubation of bovine erythrocyte ghosts with [^{32}P]NAD^+ lead to the labelling of a 55-60 kDa region of the gel. This labelling was enhanced by the addition of cysteine dependent NAD'ase: A maximum 150% increase in intensity was observed. The purified cysteine dependent NAD'ase used in these assays appeared as a single band on silver stained SDS polyacrylamide gels. It seems likely that the ADP-ribosyltransferase activity observed is due to this protein and not some contaminant. There was a linear response between the amount of cysteine dependent NAD'ase added and the amount of radiolabelled product observed and later experiments showed that the background and enzyme labelled protein had different sensitivities to treatment with mercuric acetate. However, the possibility remains that addition of the purified cysteine dependent NAD'ase adds some factor which promotes ADP-ribosylation of the 55-60 kDa protein, in a similar fashion to ADP-ribosylation of G_s by cholera toxin which requires some mammalian cytosolic protein factor termed ADP-ribosylation factor (ARF)[175].

Surprisingly, incubations containing pertussis toxin also showed increased labelling in this 55-60 kDa band as well as the expected 42 kDa protein. Cholera toxin, on the other hand reduced labelling in this region. The possibility that the apparent 55-60 kDa protein was G_i or G_s was ruled out by testing the cross reactivity of the radiolabelled band with anti-bodies raised against the C-terminal tails of G_i and G_s. The labelling of proteins catalysed by the cysteine dependent NAD'ase were not coincident with bands identified by anti-$G_i\alpha$ or anti-$G_s\alpha$ antibodies. The anti peptide antibodies were raised in rabbits using BSA as a carrier. The polyclonal antibodies recognised 64.5 kDa protein present in the assay more strongly than G_i or G_s. This protein is most likely BSA contamination from the preparation of bovine erythrocyte ghosts.

Gill and Wookalis[176] reported ADP-ribosylation of the major erythrocyte protein band III (88 kDa) by cholera toxin. ADP-ribosylation of this region was not

observed but the 76 kDa labelling is coincident with band IV (72 -78 kDa). This protein is present at relatively high concentration and therefore the labelling observed is not very specific. There are a few reports in the literature of pertussis toxin modifying protein other than than members of the trimeric G proteins; auto-ADP-ribosylation[177], tubulin[178] and most recently a 66 kDa protein in basolateral membranes[179]. ADP-ribosylation of a 28 kDa protein is seen under certain assay conditions and has been attributed to auto-ADP-ribosylation of the S1 subunit. Tubulin is a 120 kDa, made up of two subunits, α and ß of 60 kDa each. It forms part of the cytoskeleton and contains tightly bound GDP, however ADP-ribosylation of tubulin by pertussis toxin has not been shown to be physiologically relevant. These few reports suggest that ADP-ribosylation of proteins by pertussis toxin may not be as specific for the trimeric G proteins G_i, G_o and transducin as once thought.

5.4.2 Evidence for ADP-Ribosyl-Cysteine-Protein

The labelling observed could have been due to non-specific modification of susceptible residues on the target protein. Specific non-enzymatic labelling of proteins has been seen in several systems, eg ADP-ribosylation of 50-55 kDa proteins in rat liver mitochondria[96] and 36 and 39 kDa cytosolic proteins of skeletal muscle[180]. The possibility that addition of cysteine dependent NAD'ase or pertussis toxin simply increased the amount of ADP-ribose generated which then reacted with susceptible residues on the target protein was ruled out. The concentration of NAD^+ in the ADP-ribosyltransfer reaction was 2.5 μM which is sub-saturating for the cysteine dependent NAD'ase and pertussis toxin, thus NAD glycohydrolase activity would have been extremely slow. Production of ADP-ribose during the time course of the reaction would have been barely significant and incubation of the erythrocyte ghost membranes with free ADP-ribose did not cause any labelling of any protein. The possibility that the labelling observed was due to direct modification by NAD^+ remained.

Evidence that the enhanced labelling of the 55-60 kDa protein produced by the addition of cysteine dependent NAD'ase or pertussis toxin was ADP-ribosylation via a cysteine residue came from two experiments. The first showed that the labelling was sensitive to pre-treatment of the membrane proteins with NEM. This suggested that the target protein contained a reactive thiol which was susceptible to alkylation

by NEM and which was then no longer available as a site for ADP-ribosylation. This treatment also reduced the background labelling of this protein, suggesting that either the membrane preparation contained some of the purified protein whose site of action became blocked, or the membrane associated enzymes were themselves modified by NEM and were inactivated. The second experiment showed that the enzymatic labelling was released by treatment with mercuric acetate. The amount of labelling remaining after treatment of the ADP-ribosylated protein with mercuric acetate or hydroxylamine was analysed by separation of the proteins on SDS polyacrylamide gels and autoradiography. The results suggested that the background labelling was insensitive to both treatments while the enhanced labelling was sensitive to mercuric ion. The ADP-ribosylation observed may be due to labelling of different proteins of similar molecular weight or modification of the same protein, but at different sites. Transducin is an example of a protein which has more than one site for ADP-ribosylation[9]. Transducin is a peripheral membrane protein of rod outer segment of the eye and is the only member of the G protein family which can be modified by both pertussis toxin and cholera toxin.

The high background labelling observed may be due to non-specific binding of NAD^+. Mc Donald and Moss[115] have described a covalent modification of glyceraldehyde-3-phosphate dehydrogenase which was first thought to be ADP-ribosylation[114], but was actually covalent binding of NAD^+ at the active site. The preparation of ADP-ribosylated protein involved the precipitation of proteins with 5% TCA and washing of the pellet with 0.1% TCA. The background labelling is thus stable to acid, hydroxylamine and mercuric acetate. These findings rule out the possibility of modification through serine, threonine, arginine or cysteine residues. Labelling of lysine residues which are abundant on the surfaces of proteins is a possibility, which has not been tested. Modification of this groups may be distinguished by its sensitivity to treatment with weak alkali (pH 9.0). ADP-ribosylation of a modified histidine residue (diphthamide), cf diphtheria toxin or an asparagine residue, cf botulinum C3 toxin cannot be ruled out. These ADP-ribosyl linkages are stable to all the chemical treatments described and, as yet, no protocol has been determined to identify these linkages by selective chemical release.

Both NAD^+ and ADP-ribose were detected by separation on HPLC after

treatment with mercuric acetate. The NAD^+ present may have been covalently attached to the protein via a thioester linkage which was susceptible to attack by the mercuric ion. However, in these experiments the complete removal of un-reacted NAD^+ cannot be ruled out. Repeating the experiment after electroelution of the labelled product from the gel showed that only ADP-ribose was released by treatment with mercuric ion. The amount of released product was very low, and accounted for 5% of the total counts incorporated in the eluted material. The presence of SDS bound to the protein may have blocked the reaction of the mercuric ion with the thioglycosidic bond either physically, by reducing the accessibility of the ADP-ribosyl linkage to the reagent, or chemically, by reacting with the mercuric ion. Better label release may be obtained by increasing the concentration of mercuric acetate ten fold or increasing the time and the temperature of the reaction.

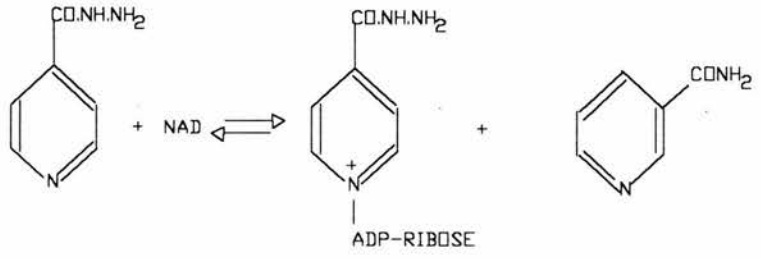
5.4.3 Inhibitors of ADP-ribosylation

Finally the effect of known inhibitors was tested. Half maximal inhibition of the cysteine dependent NAD'ase activity was observed at a concentration of 1.2 mM thymidine. This value is similar to that quoted for poly(ADP-ribose) polymerases and NAD^+: arginine ADP-ribosyltransferases (1 mM). However the specific inhibition of poly (ADP-ribose) polymerase is thought to arise from competitive inhibition of thymidine at the active site. In this case, a decrease in the concentration required to give 50% inhibition at sub-saturating substrate conditions compared to saturating conditions would be expected. This was not seen. The inhibition observed was independent of the concentration of NAD^+ in the assay, and was indicative of non-competitive inhibition, ie the inhibition was independent of whether the substrate was bound or not. The inhibition of the NAD glycohydrolase activity corresponded with potent inhibition of the ADP-ribosylation of 55-60 kDa protein observed. Thymidine inhibited background labelling to 30% of normal, and the addition of cysteine dependent NAD'ase did not produce any increase in the labelling.

Competitive inhibition was suggested by the inhibition shown by isoniazid. The concentration of inhibitor required to give 50% inhibition decreased as the concentration of NAD^+ decreased in the assay, suggesting that both molecules were competing for the same site on the enzyme. Again, inhibition of the NAD

glycohydrolase activity and inhibition of the ADP-ribosyltransferase activity was observed. Inhibition of background and enzymatic activity was observed although this was not as dramatic as the effect of thymidine. ADP-ribosylation of 60% of the protein observed in the absence of inhibitor was seen. This may seem surprising since a low concentration of NAD^+ (2.5 μM) and a high concentration of isoniazid (10 mM) were employed in the assay. If isoniazid was a competitive inhibitor of the cysteine dependent NAD'ase, then it would be expected that the enzyme activity would be completely inhibited at these concentrations of substrate and inhibitor.

Isoniazid is an analogue of nicotinamide - isonicotinic acid hydrazide. Experiments to identify product inhibition properties of nicotinamide showed that no inhibiton was obtained even at 100 fold molar excess of nicotinamide compared to substrate. Are these findings anomalous? Nakazawa *et al.*[181] proposed that isoniazid underwent a transglycosidation reaction with NAD^+. This may be catalysed



Nakazawa's Proposed Mechanism of Inhibition by Isoniazid

enzymatically with isoniazid acting as an acceptor for ADP-ribose, or ADP-ribose liberated by NAD'ase action may react directly with isoniazid in solution. The product isonicotinic acid hydrazide adenine-dinucleotide is a mimic for NAD^+ and

competes at the active site for binding. The different sensitivities of NAD'ases seen between species may be attributed to their ability to hydrolyse this modified NAD^+. The non-sensitive NAD'ases of horse and human can hydrolyse the modified NAD^+ whereas the sensitive NAD'ases of rodents and ruminants cannot and are inhibited by isoniazid (K_i 0.1 mM). This mechanism of action may be used to explain the apparent anomolies. In the ADP-ribosylation assay ADP-ribose will be transferred to the target protein in preference to isoniazid. Very little free ADP-ribose will be generated such that only low levels of inhibitor can form and thus lower than expected inhibition is observed in the ADP-ribosylation assay than expected from the NAD glycohydrolase.

The sensitivity of the cysteine dependent NAD'ase to isoniazid and thymidine is an important difference between it and pertussis toxin, which is insensitive. This property is in common with other endogenous ADP-ribosyltransferases and maybe indicates important mechanistic differences between the bacterial and mammalian enzymes. This inhibition limits the application of the purified enzyme. Potential substrates, which are present in membrane preparations containing high levels of intrinsic NAD^+ depleting enzymes may not be identified. Addition of thymidine or isoniazid will inhibit the cysteine dependent NAD'ase, and without their addition NAD^+ will not be available to the enzyme. This may have been the case for the rat hypothalamus membranes to which 10 mM isoniazid and 10 mM thymidine were added. High levels of ADP-ribosylation of G_o was observed with pertussis toxin but very little ADP-ribosylation activity was observed in the absence of pertussis toxin or the presence of cysteine dependent NAD'ase.

CONCLUSION AND FUTURE WORK

6.1 Conclusions

An endogenous cysteine specific ADP-ribosyltransferase was isolated from bovine erythrocytes by making assumptions about some of the expected properties of this enzyme by comparison with the known properties of pertussis toxin. The strategy used to purify the enzyme was based on the assumption that like pertussis toxin the enzyme would be able to use free cysteine as an acceptor for ADP-ribose. This activity could be identified as a cysteine dependent NAD'ase activity in fresh bovine erythrocyte lysate. The NAD glycohydrolase assay enabled the screening of the large number of fractions during the purification protocol and assessment of the effectiveness of the purification procedure.

In chapter three the development of a three step purification procedure to isolate this cysteine dependent NAD'ase from bovine erythrocytes was described. The purification involved, (1) precipitation in a 40 % saturated solution of ammonium sulphate, (2) binding to cysteine Sepharose and (3) binding to a dye affinity column. Following this procedure 20-50 μg of protein was purified from 200 mL of bovine blood. The purified fraction was visible with silver staining as a single band on SDS polyacrylamide gels with a relative molecular mass of 45,000. The activity had been purified 47,500 fold with a yield of 16 % of the total activity.

The cysteine dependent NAD'ase activity of the enzyme was extremely labile and was present only in fresh preparations of bovine erythrocyte lysate. The development of the successful purification strategy was dependent upon the stabilisation of this labile activity. Several factors were found to be important to solve this problem. One, the addition of 30 % ethylene glycol to all buffers following the precipitation of the activity with ammonium sulphate. Two, the addition of protease inhibitors 0.1 mM PMSF, 1 mM EDTA and 1 mM benzamidine HCl at all stages of the purification. Three, the minimisation of the time taken to purify the activity and finally the removal of reducing reagents from the purified protein on storage of the

enzyme at -16°C.

In chapter four the kinetic properties of the purified cysteine dependent NAD'ase were compared to those of pertussis toxin. The mammalian enzyme had approximately a 100 fold higher specific NAD'ase activity than pertussis toxin (1900 nmol of nicotinamide released $min^{-1}.mg^{-1}$ of protein compared to 18 $nmol.min^{-1}.mg^{-1}$). The purified cysteine dependent NAD'ase also showed higher affinity for NAD^+ (K_m value of 8 μM compared to 30 μM for pertussis toxin) and cysteine (K_m value of 4 mM compared to 100 mM). Like pertussis toxin, the cysteine dependent NAD'ase was inactive in the absence of reducing reagents and substrate kinetics were observed only for cysteine containing a free thiol group and other amino acids could not be substituted. Both enzymes had similar pH optimum curves measured at saturating concentrations of substrates suggesting that they may have a common mechanism of action. Evidence that cysteine was an acceptor for ADP-ribose was presented from the separation of the reaction products of the cysteine dependent NAD'ase incubations containing [^{3}H-adenine]NAD^+ on reverse phase HPLC. A tritium containing peak, with an elution time of 5.5 minutes was observed only in incubations containing cysteine and the cysteine dependent NAD'ase. The elution of this product between ADP-ribose and NAD^+ was consistent with it being ADP-ribose-cysteine. Treatment of the putative ADP-ribosyl-cysteine product with phosphodiesterase released AMP which confirmed the presence of mono-ADP-ribose in this product.

The ability of the cysteine dependent NAD'ase activity to ADP-ribosylate protein was confirmed in chapter five. Enhanced labelling of a 55 kDa membrane protein in bovine erythrocyte ghosts was observed. Unlike pertussis toxin, the cysteine dependent NAD'ase was unable to modify $G_i\alpha$ and was sensitive to inhibitors of poly-ADP-ribosyltransferases and NAD'ases (thymidine and isoniazid respectively). Evidence that mono-ADP-ribosylation of a cysteine residue on the target protein had nonetheless occurred was presented. Firstly, the labelling was sensitive to pre-treatment of the membranes with NEM; a sulphydryl alkylating reagent. Secondly, the labelling was released by treatment with mercuric ion and was resistant to neutral hydroxylamine. Thirdly, separation of the products of the treatment of labelled protein with mercuric ion on ion exchange HPLC showed that the major product had an elution time coincident with that of ADP-ribose. These results suggested that the

cysteine dependent NAD'ase purified from bovine erythrocytes also had a novel NAD^+: cysteine mono-ADP-ribosyltransferase activity.

The strategy employed to purify the cysteine dependent NAD'ase has identified an enzyme activity which in terms of its NAD'ase activity is similar to pertussis toxin but which exhibits a novel NAD^+: cysteine ADP-ribosyltransferase activity. ADP-ribosylation of a 55 kDa protein in bovine erythrocytes has not been reported previously. The methodologies developed for the purification, and the initial characterisation of the kinetic properties and ADP-ribosyltransferase activities of this enzyme form the ground work upon which a more detailed characterisation of the enzyme and the target protein could be based.

6.3 Future Work

Further characterisation of the novel target protein was not feasible for this present study, due to the constraints of time and expense of [^{32}P-adenylate]NAD^+. Ideally confirmatory amino acid sequence would be obtained and a data base search run to identify the target protein. Purification of the target protein would be required to separate the enzyme and background labelled proteins; assuming that different proteins of similar molecular weight are modified and not different sites on the same protein. For example a two dimensional gel electrophoresis system could be employed. Native ADP-ribosylated membrane proteins could be separated on the basis of charge in the first dimension and denatured molecular weight in the second. The separated protein could then be blotted onto polyvinylidene difluoride (PVDF) membranes. The protein may be sequenced directly from this inert sequence compatible membrane. If the protein was N-terminally blocked it may be digested *in situ* with protease and the peptides separated on reverse phase HPLC[182]. If a known protein of known function was identified from the target protein sequence, then the presence of its activity in the erythrocyte ghost could be tested, and the effect of ADP-ribosylation on the target protein assessed.

An attempt to characterise the distribution of the enzyme was made by raising antibodies against the cysteine dependent NAD'ase in mice. This proved unsuccessful as the antibodies raised did not recognise denatured antigen on Western blots. This work should be repeated. An alternative approach, involving the linking of the antigen to a carrier, to increase the likelihood of success could be employed. The antigen could be blotted onto nitrocellulose, and the nitrocellulose bound antigen then used to immunise a mouse. The advantage of this approach would be that denatured material from Western blotting could be used to raise antibodies, and therefore increase the chances of reaction with the antigen after Western blotting.

An important advance in the understanding of the role of endogenous ADP-ribosyltransferases could be made from the characterisation of the protein at the molecular level. If the full amino acid sequence were known then better comparison with the bacterial toxins could be made, and related proteins present in complex systems may be identified, that otherwise would be difficult to identify enzymatically. To do this amino acid sequencing of the N-terminal or of an internal peptide is required. Oligonucleotide probes, coding for the identified sequence may then be synthesised and used to screen a cDNA library in order to isolate the nucleotide sequence coding for the enzyme activity.

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